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(54) Title: SMALL MOLECULE INHIBITORS OF BCL–2 PROTEINS

(57) Abstract

Small molecule inhibitors of Bcl–2 function are used to induce apoptosis of cells which are subject to Bcl–2, which cells are otherwise subject to Bcl–2 mediated blockage of apoptosis. The compounds are useful for treating cancer, autoimmune disorders and viral infection.

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SMALL MOLECULE INHIBITORS OF BCL-2 PROTEINS

Cross-Reference to Related Applications

This application claims priority from provisional applications Ser. No. 60/128,100, filed April 7, 1999, and 60/093,561, filed July 21, 1998, the entire disclosures of which are incorporated herein by reference.

Field of the Invention

The present invention generally relates to the field of oncology and inhibitors of Bcl-2 proteins, and more particularly to small molecule inhibitors of Bcl-2 proteins involved in mediating the death of cancer cells, virally infected cells and self-reactive lymphocytes.

Background of the Invention

Bcl-2 (B cell lymphoma/leukemia 2) was originally identified at the chromosomal breakpoint of t(14;18)-bearing B-cell lymphomas. Bcl-2 is now known to belong to a growing family of proteins which regulate programmed cell death or apoptosis. The Bcl-2 family includes both death antagonists (Bcl-2, Bcl-x_L, Bcl-w, Bfl-1, Brag-l, Mcl- I and Al) and death agonists (Bax, Bak, Bcl-x_S, Bad, Bid, Bik and Hrk) (Thompson, *Science* 267:1456-62 (1992); Reed, *J. Cell Biol.* 124:1-6 (1994); Yang *et al.*, *Blood* 88:386-401 (1996)). This family of molecules shares four homologous regions termed Bcl homology (BH) domains BH 1, BH2, BH3, and BH4. All death antagonist members contain the BH4 domain while the agonist

members lack BH4. It is known that the BHI and BH2 domains of the death antagonists such as Bcl-2 are required for these proteins to heterodimerize with death agonists, such as Bax, and to repress cell death. On the other hand, the BH3 domain of death agonists is required for these proteins to heterodimerize with Bcl-2 and to promote apoptosis.

Programmed cell death or apoptosis plays a fundamental role in the development and maintenance of cellular homeostasis. Homologous proteins and pathways in apoptosis are found in a wide range of species, indicating that cellular demise is critical for the life and death cycle of the cell in all organisms. When extracellular stimuli switch on the cell-death signal, the response of the cell to such stimuli is specific for the particular cell type (Bonini *et al.*, *Cell* 72:379-95 (1993)). The pathway to cellular suicide is controlled by certain checkpoints (Oltvai, *Cell* 79:189-92 (1994)). The Bcl family proteins, including both antagonists of apoptosis (such as Bcl-2) and agonists of apoptosis (such as Bax), constitute the primary checkpoint. As such, the transmission of a cell-death signal can be either promoted or blocked by the different combinations of the Bcl-2 family members. The three-dimensional structure of a death antagonist, Bcl-X_L, as determined by X-ray crystallography and NMR spectroscopy, provides a structural basis for understanding the biological functions of Bcl-2 family members and for developing novel therapeutics targeting Bcl-2 mediated apoptotic pathways (Muchmore *et al.*, *Nature* 381:335-41 (1996)).

The detailed mechanism of Bcl-2 proteins in mediating molecular pathways of apoptosis has been the subject of intensive investigation. It is known that the apoptotic signaling pathway involves the activation of caspases which, once activated, cleave several cellular substrates such as poly(adenosine diphosphate-ribose) polymerase (PARP) and lead to final events of apoptosis. Bcl-2 plays a crucial role in regulating the process of apoptosis. One possible mechanism for Bcl-2 function is that Bcl-2 inhibits the release of cytochrome c from mitochondria. Cytochrome c is important for the activation of caspases.

As such, Bcl-2 blocks caspase activation and subsequent events leading to apoptosis.

Being able to block apoptosis, Bcl-2 is known to contribute to neoplastic cell expansion by preventing normal cell turnover caused by physiological cell death mechanisms. High levels and aberrant patterns of Bcl-2 gene expression are found in a wide variety of human cancers, including ~30-60% of prostate, ~90% of colorectal, ~60% of gastric, ~20% of non-small cell lung cancers, ~30% of neuroblastomas, and variable percentages of melanomas, renal cell, and thyroid cancers, as well as acute and chronic lymphocytic and non-lymphocytic leukemias (Ellis *et al.*, *Cell Biol.* 7, 663 (1991); Henkart, *Immunity* 1, 343 (1994)); Kägi *et al.*, *Science* 265, 528 (1994); Kägi *et al.*, *Nature* 369, 31 (1994); Heusel *et al.*, *Cell* 76, 977 (1994)).

The expression levels of Bcl-2 protein also correlate with relative resistance to a wide spectrum of current chemotherapeutic drugs and γ-irradiation (Hanada *et al.*, *Cancer Res.* 53:4978-86 (1993); Kitada *et al.*, *Antisense Res. Dev.* 4:71-9 (1994); *Miyashita et al.*, *Cancer Res.* 52:5407-11 (1992); *Miyashita et al.*, *Blood* 81:151-7 (1993)). Since Bcl-2 can protect against such a wide variety of drugs which have very different mechanisms of action, it is possible that all these drugs use a common final pathway for the eventual induction of cell death which is regulated by Bcl-2. This notion is supported by the findings that chemotherapeutic drugs induce cell death through a mechanism consistent with apoptosis as opposed to necrosis. Therefore, Bcl-2 can inhibit the cell killing effect of currently available anticancer drugs by blocking the apoptotic pathway.

Because of its role in blocking apoptosis, Bcl-2 plays an important role in many types of cancer. As noted above, Bcl-2 blocks apoptosis, thereby preventing normal cell turnover. As a result, neoplastic cell expansion occurs unimpeded by the normal cellular turnover process. Prostate cancer is one particular example where Bcl-2 has important implication in the pathogenesis and treatment for a disease. Approximately

100,000 new cases of prostate cancer are diagnosed each year in the United States and about 30,000 deaths per year are attributable to this disease (Lynn et al., *JNCI* 87:867 (1995)). It has recently been found that hormone therapy-resistant prostate cancers express Bcl-2 (McDonnell *et al.*, *Cancer Res.* 52:6940-4 (1992)), while the normal prostate cells from which prostate cancers originate lack Bcl-2 (Colombel *et al.*, *Am J Pathol* 143:390-400 (1993)). This indicates that Bcl-2 may protect prostate cancer cells from undergoing apoptosis induced by the anticancer drugs, such as Taxol (Haldar *et al.*, *Cancer Res.*, 56:1235-5 (1996)). The clinical efficacy of nearly every cytotoxic anticancer drug currently available depends directly or indirectly on the assumption that tumor cells grow more rapidly than normal cells. However, this may not apply to human prostate cancer cells, which show very slow growth kinetics. Tumor kinetics studies have indicated that prostate cancer may be the consequence of the imbalance in cell turnover mechanisms more so than an increase in cell cycle rates. Thus, current anticancer drugs may not be effective in eradicating these nonproliferative prostate cancer cells.

The understanding of the biology of Bcl-2 in cancer and chemoresistance has opened new avenues in the development of novel anticancer strategies. One effective approach to overcome the chemoresistance of prostate cancers is to inhibit the protective function of Bcl-2 proteins. New drugs that modulate Bcl-2 mediated apoptotic response would represent a novel mechanism-based strategy for the treatment of prostate cancers and other cancers. Because the function of Bcl-2 is not absolutely necessary in many normal cell types (Veis *et al.*, *Cell*, 75:229-40 (1993)), a systematic inhibition of Bcl-2 may not affect the normal cellular function. This notion is supported by recent encouraging data from the clinical trial that antisense oligonucleotides targeted against the Bcl-2 gene can specifically inhibit non-Hodgkin's lymphoma in humans (Webb *et al.*, *Lancet* 349:1137-41 (1997)). However, the clinical value of such antisense oligonucleotides is limited by their lack of enzymatic stability, cell

permeability, and oral activity. As discussed above, currently available anticancer drugs may not be effective due to the chemoresistance of prostate cancer cells. Therefore, there is an impending need for highly potent, cell permeable, and orally active Bcl-2 inhibitors as a new generation of effective therapeutics for the treatment of prostate cancer, as well as other cancers.

Compared to other therapeutics such as antibodies, peptides or antisense oligonucleotides, small organic drugs may possess several advantages in the clinical application: (1) they are less likely to be immunogenic; (2) they are likely to be stable and to be able to cross the cell membrane; (3) they are more likely to be administrable through the oral route, which is most desirable in terms of patient compliance; and (4) they are amenable to synthesis and modification which significantly lowers the cost of the therapeutic treatment.

Summary of the Invention

It is an object of the invention to provide small molecule inhibitors of bcl-2 function useful in treatment of cancer, autoimmune disease and certain types of viral infection which are characterized by cellular signals which inhibit apoptosis.

It is an object of the invention to induce apoptosis of cells, particularly cancer cells, most particularly cancer cells which are regulated by Bcl-2.

It is an object of the invention to provide novel therapeutics and methods of treatment for reversing Bcl-2-mediated blockage of cell apoptosis in cancer cells.

It is an object to provide of the invention to overcome Bcl-2-mediated chemoresistance in tumor cells.

These and other objects of the invention are apparent from the following description.

A method of inducing apoptosis of cells regulated by Bcl-2 in a subject is provided. An effective amount of an active compound is administered to the subject. Preferably, the compound causes the fragmentation of DNA in a Bcl-2 transfected HL-60 cell line when incubated with such cells at a concentration of not more than 100 μM for 24 hours. In some embodiments, the compound is also characterized by a dissociation constant K_D of not more than about 500 μM, preferably no more than about 100 μM, most preferably no more than about 10 μM, for binding the hydrophobic pocket on the Bcl-2 protein formed by the BH1, BH2, and BH3 domains.

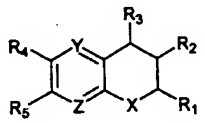
By "regulated by Bcl-2" with respect to the condition of a cell is meant that the balance between cell proliferation and apoptotic cell death is controlled, at least in part, by Bcl-2. By "apoptotic cell death" is meant the programed death which results in controlled autodigestion of the cell, as opposed to necrotic cell death. Apoptotic cell death is characterized by cytoskeletal disruption, cell shrinkage, and membrane blebbing. The nucleus undergoes condensation and nuclear DNA becomes degraded and fragmented. Apoptosis is also characterized by loss of mitochondrial function. Necrotic cell death, on the other hand, is a pathological form of cell death resulting from acute cellular injury, which is typified by rapid swelling and lysis.

According to certain embodiments of the invention, the cells induced to undergo apoptosis comprise cancer cells, virus-infected cells or self-reactive lymphocytes. Thus, the active compounds are used to treat cancer, viral infection, or autoimmune disorders.

In another embodiment, a method of reversing Bcl-2-mediated blockage of apoptosis in cancer cells is provided by contacting such cells with an active compound of the invention. In another embodiment, a method is provided for treating a subject afflicted with a cancer characterized by cancer cells which express Bcl-2. The method comprises administering an effect amount of an active compound of the invention.

Active compounds which have a molecular weight in the range of from about 150 to about 500 daltons.

According to one embodiment of the invention, the compounds have the formula I:



I

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; O; and S;

Y and Z are independently selected from the group consisting of CH and N; and when Z is N, then Y may further be $-CR_6$, where R_6 is selected from the group consisting of CH_3; OCH_3; CNH_2; and COH;

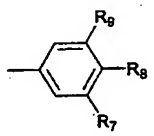
R_1 is selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; $NH(C_{1-3}$ alkyl); $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$: $NHNHCOCH_3$; $NHNHCONH_2$; phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings, preferably a heterocyclic ring selected from the group consisting of piperidino, piperazino, morpholino, pyrimidyl, pyrrolidino and imidazo;

R_2 is selected from the group consisting of hydrogen; C_{1-3} alkyl; C_{1-3} alkoxy; halogen; CF_3; NH_2; OH; COOH; $COOCH_3$; $CONH_2$; and $CONHCH_3$;

or, R_1 and R_2 together may form the group -$CH_2CH_2CH_2$- or -$CH_2CH_2CH_2CH_2$-;

or, R_1 and R_2 together may form, starting from R_1, the group -$NHCH_2CH_2$-, -$NHCOCH_2$-, or -$OCOCH_2$-;

R_3 is selected from the group consisting of H; CH_3; CF_3; OCH_3; NH_2; OH; COOH; $COCH_3$; $CH{=}CH_2$; $CH_2{=}CHCH_2$; $CH(CH_3)_2$; CH_2OH; CH_2NH_2; CH_2COOH; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, OCH_3 or CF_3; five- and six-member heterocyclic rings, preferably a heterocyclic ring selected from the group consisting of piperidinyl, piperazinyl, morpholino, pyrimidyl, pyrrolyl, pyrrolidino, and imidazyl; and a substituted phenyl group of the formula:



wherein

R_7, R_8 and R_9 are independently selected from the group consisting of hydrogen, CH_3, CF_3, OH, OCH_3, CH_2OH and CHO; provided that at least two of the members of the group R_7, R_8 and R_9 must be OH or OCH_3 when the remaining member of the group is hydrogen, CH_3 or CF_3;

R_4 and R_5 are independently selected from the group consisting of hydrogen, CH_3, and OCH_3; and when Y and Z are both CH, R_4 and R_5 may be further selected from OH and NH_2;

or, R_4 and R_5 together may form the group -$CH_2CH_2CH_2$- or -$CH_2CH_2CH_2CH_2$-;

or, R_4 and R_5 together may form, starting from R_4, the group -$NHCH_2CH_2$-, -$NHCOCH_2$-, -$OCOCH_2$- or -$O(CH_2)_nO$-, wherein n is 1, 2 or 3;

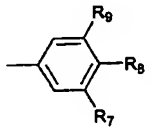
or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

Preferably, R_2 is CH_3, CH_2CH_3, COOH, $COOCH_3$, $CONH_2$, or $CONHCH_3$.

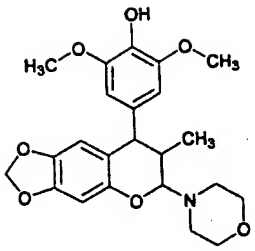
Preferably, R_7, R_8 and R_9 are all OCH_3; or R_7 and R_9 are OCH_3, and R_8 is OH.

When R_1 or R_3 is substituted cyclohexyl, the preferred position of the substitution is *para*. Likewise, when R_1 is substituted phenyl, the preferred position of the substitution is *para*.

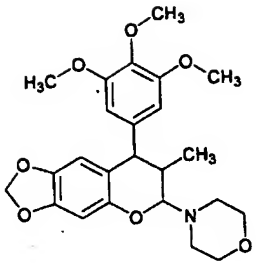
The preferred group corresponding to R_3 in formula I is the substituted phenyl group of the formula:



Preferred compounds according to formula I include the compounds identified as HA11-1 through HA11-73, listed in Table 1, below. Most preferred compounds according to formula I include HA11-57 and HA11-17:

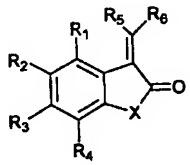


HA11-57



HA11-17

According to another embodiment of the invention, the active compounds have the formula II:



II

wherein

R_1, R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; and $NH(C_{1-3}$ alkyl); and one of R_1, R_2, R_3 and R_4 may be phenyl or a heterocyclic ring, preferably a heterocyclic ring selected from the group consisting of piperidino, piperazino, morpholino, pyrimidyl, pyrrolidino and imidazo; provided at least one of R_1, R_2, R_3 and R_4 must be hydrogen;

R_5 and R_6 are independently selected from the group consisting of hydrogen; CN; CH_2CN; $COOCH_3$; $CONH_2$; phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CH_3, OCH_3, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings, preferably a heterocyclic ring selected from

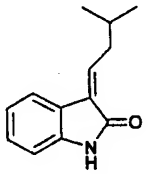
the group consisting of pyrrolyl, imidazolyl, piperidinyl, piperazinyl, morpholino, pyrimidyl and pyrrolidino; provided, only one of R_5 or R_6 may be phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic in the same compound, and further provided that when one of R_5 or R_6 is phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic, then the other must be hydrogen;

or at least one of R_5 and R_6 may be halogen, provided that the other must be C_{1-5} alkyl or C_{1-5} alkoxy.

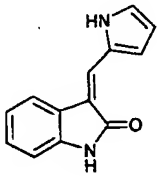
or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

When R_5 or R_6 is substituted phenyl or substituted cyclohexyl, in formula II, the preferred position of the substitution is *para*.

Preferred compounds according to formula II include the compounds identified as HA12-3 and HA12-16 (compound HA12-16 may also be identified herein as "HA01"):



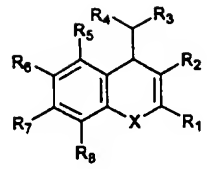
HA12-3



HA12-16

In the compounds of formula I and II, where halogen substitution is possible, chorine, fluorine and bromine are preferred, with fluorine being most preferred.

According to another embodiment of the invention, the active compounds have the formula III:



III

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; NCH_3; O; and S;

R_1 is selected from the group consisting of OH; NH_2; CHO; $COCH_3$; COOH; $N(C_{1-3}$ alkyl$)_2$; $NH(C_{1-3}$ alkyl); $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $N(C_{1-3}$alkyl$)_2$; $NH(C_{1-3}$ alkyl); and five- and six-member heterocyclic rings, preferably a heterocyclic ring selected from the group consisting piperidinyl, piperazinyl, morpholino, pyrimidyl, pyrrolyl, pyrrolidino and imidazyl;

R_2 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; OH; NH_2; CHO; $COCH_3$; $OCOCH_3$; $OCOCH_2CH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$;

R_3 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; COOH; $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $CH=CH_2$; $CH_2CH=CH_2$; CH_2CHO; and five- and six-member heterocyclic rings, preferably a heterocyclic ring selected from the group consisting piperidinyl, piperazinyl, morpholino, pyrimidyl, pyrrolyl, pyrrolidino and imidazyl;

R_4 is selected from the group consisting of C_{1-3} alkyl; C_{1-3}alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; $COCH_3$; COOH;

$COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$; $OCOCH_3$; $OCOCH_2CH_3$;

R_5 is selected from the group consisting of hydrogen CH_3; OCH_3; OH: NH_2; Br; Cl; and F; and

R_6, R_7 and R_8 are selected from the group consisting of hydrogen, CH_3; CH_2CH_3; CF_3; NH_2; OH; OCH_3; CN; NO_2; Cl; Br; F; COOH; and $COOCH_3$; provided, at least one member of the group R_6, R_7 or R_8 must be Cl, Br or F when the remaining members of said group are hydrogen;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

Preferred for formula III are the following:

R_1: NH_2; $N(C_{1-3}$ alkyl$)_2$; and $NH(C_{1-3})$ alkyl; piperidinyl; piperazinyl; morpholino; pyrimidyl; pyrrolyl; pyrrolidino; and imidazyl;

R_2: $COCH_3$; $OCOCH_3$; $OCOCH_2CH_3$; COOH; $COOCH_3$; $COOCH_2CH_3$; and $COOCH_2CH_2CH_3$;

R_3: CN; CH_2CN; CH_2NO_2; $CH{=}CH_2$; $CH_2CH{=}CH_2$; and CH_2CHO;

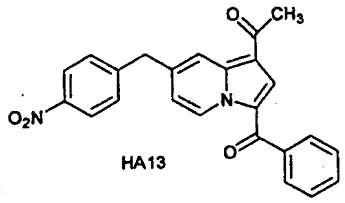
R_4: $COCH_3$; $OCOCH_3$; $OCOCH_2CH_3$; COOH; $COOCH_3$; $COOCH_2CH_3$; and $COOCH_2CH_2CH_3$;

R_5: hydrogen, Br; Cl; and F;

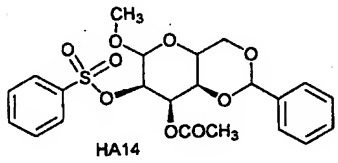
R_6, R_7 and R_8: NH_2; OH; OCH_3; CN; NO_2; Cl; Br and F.

When R_6, R_7 or R_8 are Br, Cl or OCH_3, the preferred positions of the substitution are R_6 and R_8.

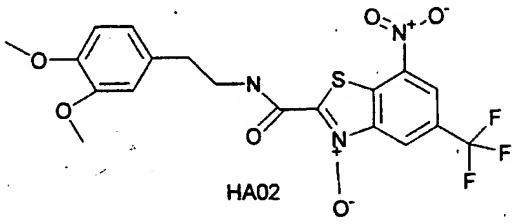
According to another embodiment of the invention, the active compound for use in the method of the invention is selected from the group consisting of compounds HA13, HA14, HA02, HA03 and HA04:



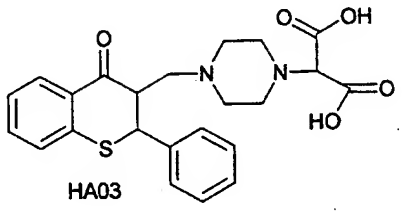
HA13



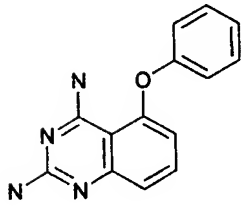
HA14



HA02



HA03



HA04

Description of the Figures

Fig. 1 is a plot of the binding of fluorescein-labeled peptide 1193 with Bcl-2 protein.

Fig. 2 is a graph of the binding interaction of fluorescein-labeled peptide 1193 (Flu-1193) with Bcl-2 protein (Bcl2:Flu-1193) and other proteins such as Bax, CD4, and the SH3 domain of the Bcr-Abl oncoprotein. Fig. 2 also shows the binding of Bcl-2 protein to fluorescein-labeled peptides derived from CD4 (Flu-1250 and Flu-1251) and Bcr-Abl SH3 (Flu-1217). The lack of binding interaction detected in these control systems (the signals were close to the background level of free Flu-1193 (Flu-1193 alone)), demonstrates the specificity of the binding of Flu-1193 to Bcl-2.

Fig. 3 is a DNA fragmentation assay of HL-60 cells transfected to overexpress Bcl-2 and treated with compound HA13, HA14 or HA11-57: lane 0, control; lane 1, HA13; lane 2, HA14; lane 3, HA11-57.

Fig. 4 is a DNA fragmentation assay of 697 cells treated with compound HA01, HA02 HA04, Taxol or negative control compound having no affinity for Bcl-2.

Fig. 5 is a DNA fragmentation assay of HL-60 cells transfected to overexpress Bcl-2 and treated with compound HA14-1. Lane A: cells treated with HA14-1; lane B, cells pretreated with fluoromethyl ketone, then treated with HA14-1.

Fig. 6 is a graph of an assay measuring the binding of compound HA14-1 to Bcl-2 protein.

Detailed Description of the Invention

A computer screening technique has been employed to discover a novel class of small organic compounds as potent Bcl-2 inhibitors and new anticancer agents. The three dimensional structure of Bcl-2 was constructed based on the X-ray and NMR structure of the highly homologous protein Bcl-x_L (>98% sequence homology to Bcl-2 in the four functionally important BH domains) published by others (McDonnell *et al.*,

Cancer Res. 52:6940 (1992); Hague *et al., Oncogene* 9, 3367 (1994); Castle *et al., Am. J. Pathol* 143, 1543 (1993); Littman, *Curr. Biol.* 4:618 (1994)). A hydrophobic binding pocket was found in the structure of Bcl-2 which is formed by the BH1, BH2, and BH3 domains. A highly sensitive Bcl-2 ligand binding assay was then employed to test these compounds for specific binding to the hydrophobic surface pocket. This pocket is required for the anti-apoptotic function of Bcl-2; a variety of mutations at his site have been shown to inhibit function of Bcl-2 proteins (Yin *et al.*, *Nature* 369:321-3, 1994).

Molecular Modeling

DOCK3.5 is an automatic algorithm to screen small-molecule databases for ligands that could fit a given receptor. Meng *et al.*, *J. Comp. Chem.* 15:505 (1992). The program exploits a geometric description of the surface of the target molecule to define plausible binding pockets. To exploit this approach, a "negative image" of the ligand binding pocket on the protein surface is created. The image is created by the computational equivalent of placing atom-sized spheres into the binding pocket. A representative set of spheres are identified by DOCK3.5 that fit extremely well into the binding pocket. The generated spheres constitute an irregular grid that is matched to the atomic centers of potential ligands. The list of atom centers, or more conveniently the matrix of interatomic distances linking these atom centers forms a useful description of the binding site. The matrix of interatomic distances for the putative ligand is also made. The best mutual overlap of the two matrices is sought. This alignment specifies the orientation of the ligand relative to the negative image of the protein and thus docks the ligand into the protein's binding pocket.

The DOCK3.5 compound was used to screen the 150,000 compounds contained in the Available Chemicals Dictionary (Molecular Design Limited, San Leonadro, CA) as potential ligands for the Bcl-2 binding pocket. Both shape complementarity and electrostatic interactions with the

Bcl-2 binding pocket were used as scoring criteria. On a computer, these compounds were then visually screened three times independently in the context of the Bcl-2 binding pocket. The result is the compounds compiled in Table 1. Screening also identified compounds HA02, HA03 and HA04. The compounds identified as "RCL __" in Table I are available from Molecular Design Limited. Compounds HA02, HA03 and HA04 are also commercially available.

Table I Compounds for formula I

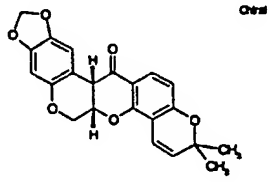
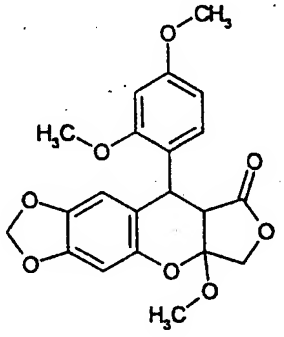
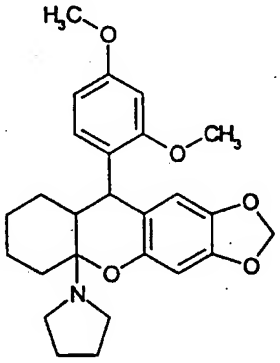
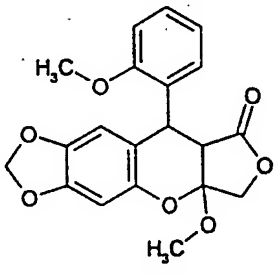
Comp. Number	MOLNAME	M.W	MOLSTRUCTURE
HA11-1	MILLETTONE	378.378	
HA11-2	9-(2,4-DI-MEO-PH)-5A-MEO-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA(B,G)NAPHTHALEN-8-ONE	400.381	
HA11-3	1-(10-(2,4-DIMETHOXY-PH)-1,3,5-TRIOXA-CYCLOPENTA(B)ANTHRACEN-5A-YL)-PYRROLIDINE	437.533	
HA11-4	5A-MEO-9-(2-MEO-PH)-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA(B,G)NAPHTHALEN-8-ONE	370.355	

Table I Compounds for formula I

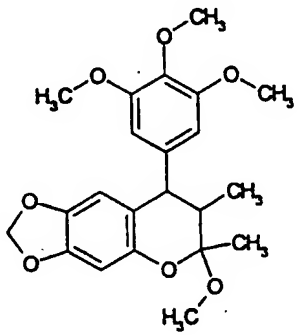
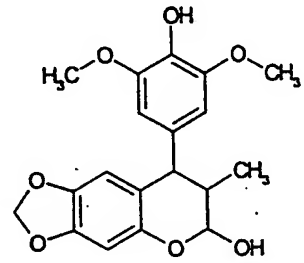
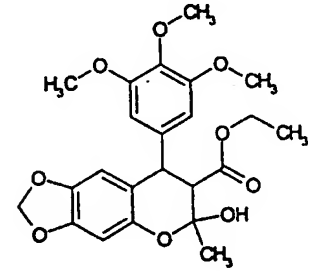
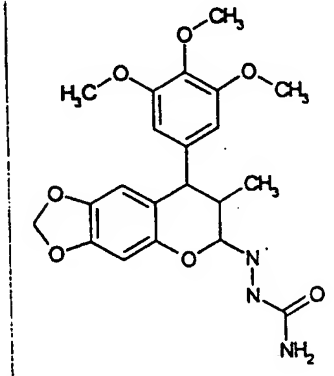
HA11-5	6-MEO-6,7-DIMETHYL-8-(3,4,5-TRIMETHOXY-PH)-7,8-2H-6H-(1,3)DIOXOLO(4,5-G)CHROMENE	402.44	
HA11-6	8-(4-HO-3,5-DIMETHOXY-PH)-7-ME-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	360.36	
HA11-7	6-HO-6-ME-8-(TRI-MEO-PH)-[1,3]DIOXOLO[4,5-G]CHROMENE-7-CARBOXYLIC ACID ET ESTER	446.449	
HA11-8	RCL R17,027-5	431.443	

Table I Compounds for formula I

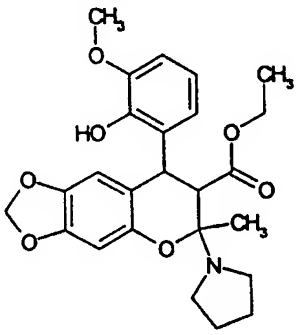
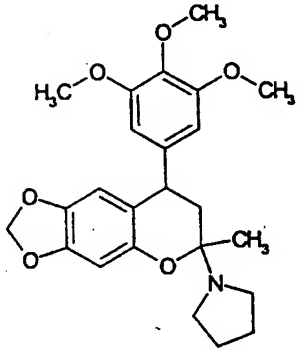
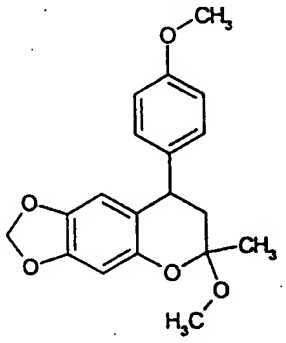
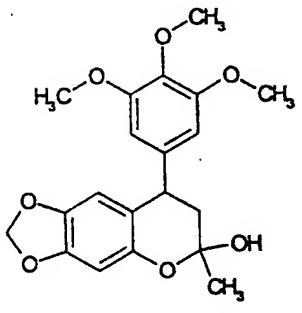
HA11-9	RCL R17,028-3	455.504	
HA11-10	1-[6-ME-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	427.494	
HA11-11	6-MEO-8-(4-METHOXY-PHENYL)-6-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	328.362	
HA11-12	6-ME-8-(3,4,5-TRIMETHOXY-PHENYL)-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	374.387	

Table I Compounds for formula I

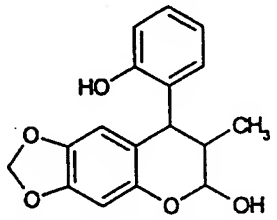
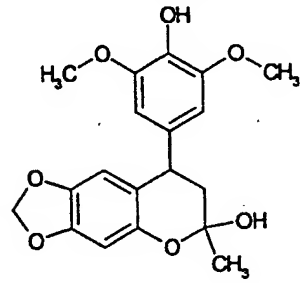
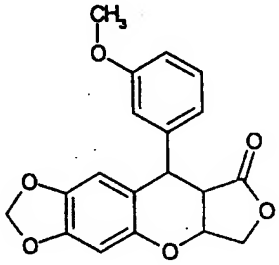
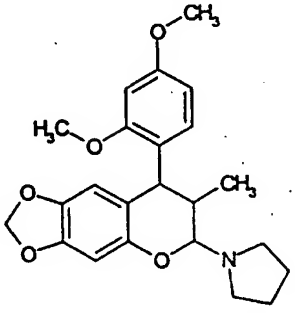
HA11-13	8-(2-HYDROXY-PHENYL)-7-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	300.308	
HA11-14	8-(4-HO-3,5-DIMETHOXY-PH)-6-ME-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	360.36	
HA11-15	9-(3-MEO-PH)-5A,6,8A,9-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA[B,G]NAPHTHALEN-8-ONE	340.329	
HA11-16	1-[8-(2,4-DI-MEO-PH)-7-ME-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	397.468	

Table I Compounds for formula I

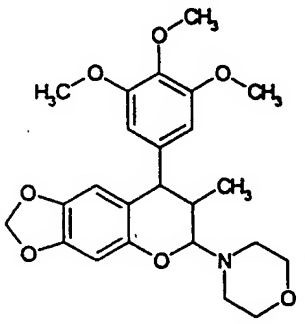
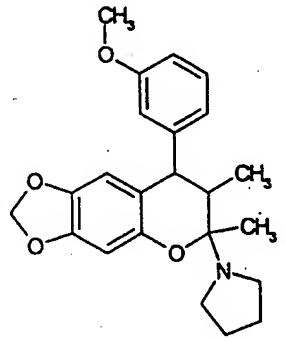
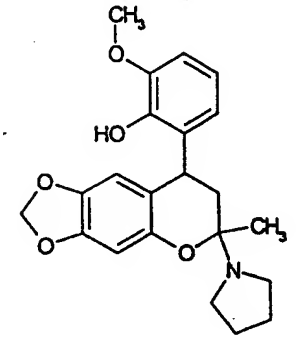
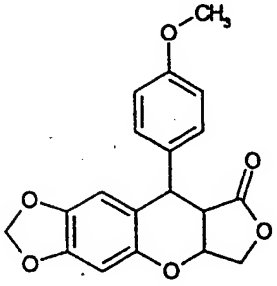
HA11-17	4-[7-ME-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-MORPHOLINE	443.493	
HA11-18	1-[8-(3-MEO-PH)-6,7-DI-ME-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	381.469	
HA11-19	2-MEO-6-(6-ME-6-PYRROLIDIN-1-YL-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	383.441	
HA11-20	9-(4-MEO-PH)-5A,6,8A,9-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA[B,G]NAPHTHALEN-8-ONE	340.329	

Table I Compounds for formula I

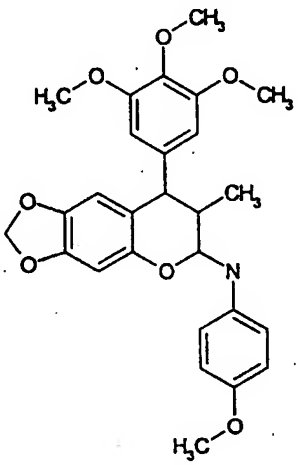
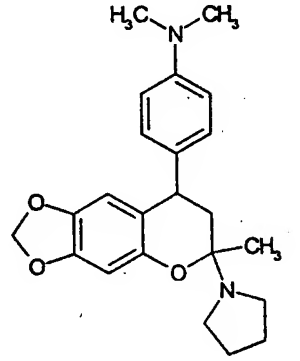
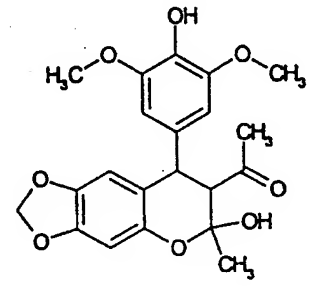
HA11-21	(4-MEO-PH)-[7-ME-8-(3,4,5-TRI-MEO-PH)-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-AMINE	479.526	
HA11-22	DI-ME-[4-(6-ME-6-PYRROLIDIN-1-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PH]-AMINE	380.485	
HA11-23	1-[6-HO-8-(4-HO-3,5-DI-MEO-PH)-6-ME-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-7-YL]-ETHANONE	402.397	

Table I Compounds for formula I

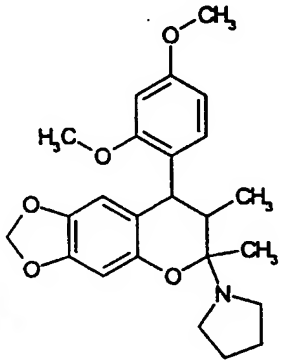
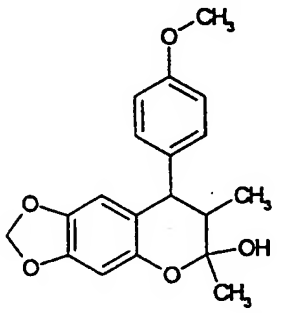
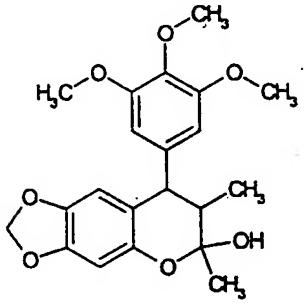
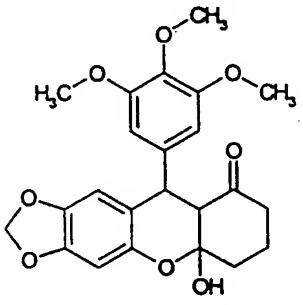
HA11-24	1-[8-(2,4-DI-MEO-PH)-6,7-DI-ME-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	411.495	
HA11-25	8-(4-METHOXY-PHENYL)-6,7-DIMETHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	328.362	
HA11-26	6,7-DIMETHYL-8-(3,4,5-TRIMETHOXY-PH)-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	388.414	
HA11-27	5A-HO-10-(3,4,5-TRI-MEO-PH)-HEXAHYDRO-1,3,5-TRIOXA-CYCLOPENTA[B]ANTHRACEN-9-ONE	428.435	

Table I Compounds for formula I

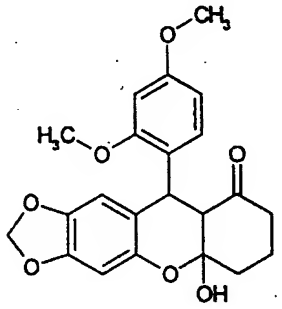
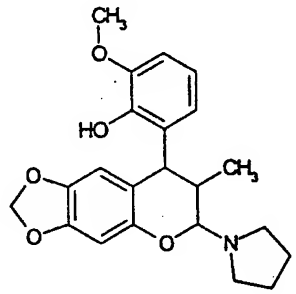
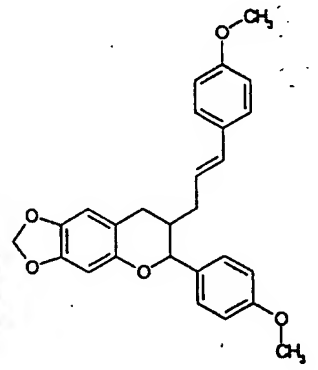
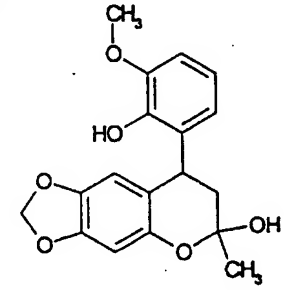
HA11-28	10-(2,4-DIMETHOXY-PH)-5A-HO-HEXAHYDRO-1,3,5-TRIOXA-CYCLOPENTA[B]ANTHRACEN-9-ONE	398.409	
HA11-29	2-MEO-6-(7-ME-6-PYRROLIDIN-1-YL-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	383.441	
HA11-30	6-(4-MEO-PH)-7-[3-(4-MEO-PH)-ALLYL]-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	430.497	
HA11-31	8-(2-HO-3-MEO-PHENYL)-6-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	330.334	

Table I Compounds for formula I

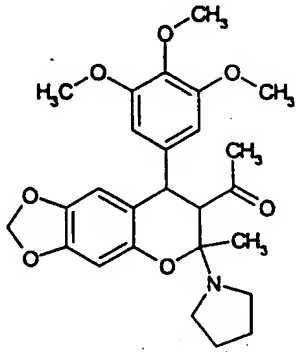
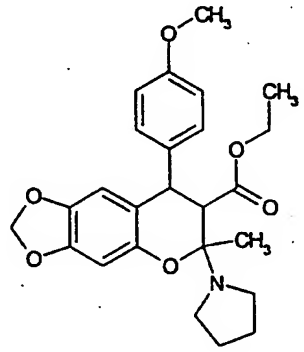
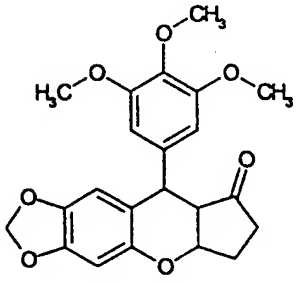
HA11-32	RCL R17,093-3	469.531	
HA11-33	RCL R17,094-1	439.505	
HA11-34	9-(3,4,5-TRI-MEO-PH)-4H-5AH-1,3,5-TRIOXA-DICYCLOPENTA[B,G]NAPHTHALEN-8-ONE	398.409	

Table I Compounds for formula I

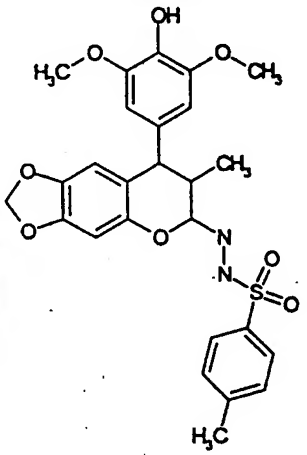
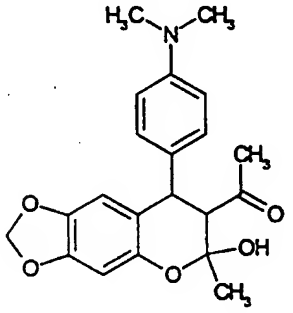
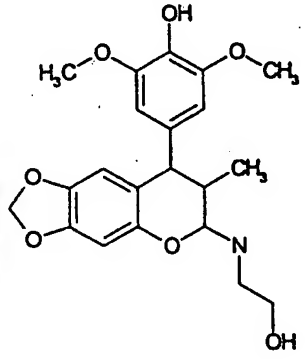
HA11-35	RCL R17,0976	528.579	
HA11-36	1-[8-(4-DI-ME-AMINO-PH)-6-HO-6-ME-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-7-YL]-ETHANONE	369.415	
HA11-37	RCL R17,106-9	403.428	

Table I Compounds for formula I

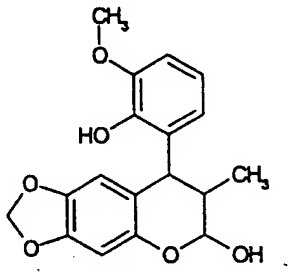
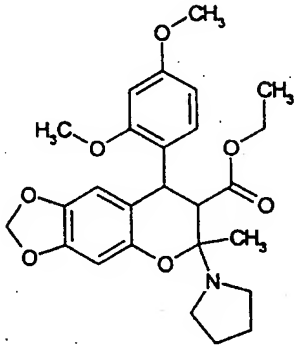
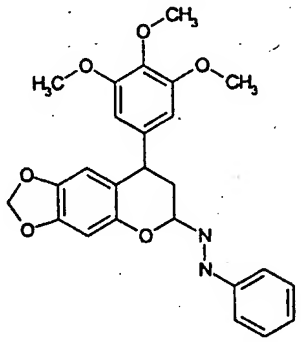
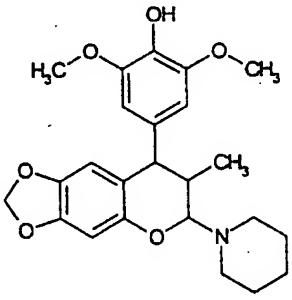
HA11-38	8-(2-HO-3-MEO-PHENYL)-7-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	330.334	
HA11-39	RCL R17,118-2	469.531	
HA11-40	N-PH-N'-[8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-HYDRAZINE	450.488	
HA11-41	2,6-DI-MEO-4-(7-ME-6-PIPERIDIN-1-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	427.494	

Table I Compounds for formula I

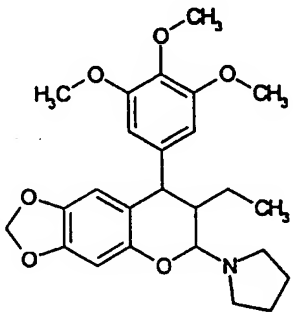
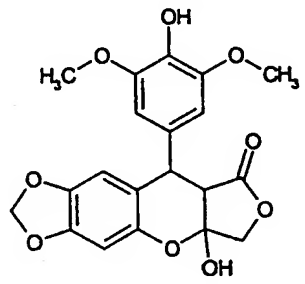
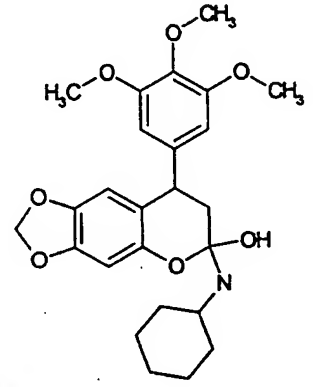
HA11-42	1-[7-ET-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	441.521	
HA11-43	5A-HO-9-(HO-3,5-DI-MEO-PH)-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA[B,G]NAPHTHALEN-8-ONE	402.353	
HA11-44	6-CYCLOHEXYLAMINO-8-(3,4,5-TRI-MEO-PH)-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	457.52	

Table I Compounds for formula I

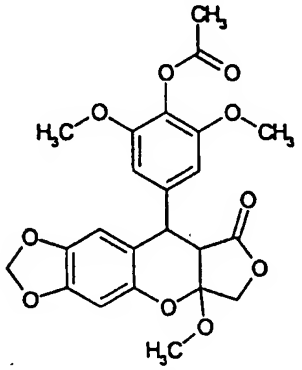
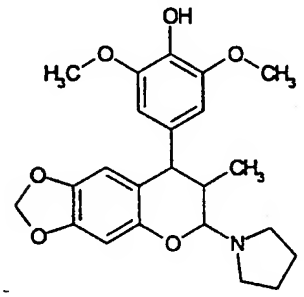
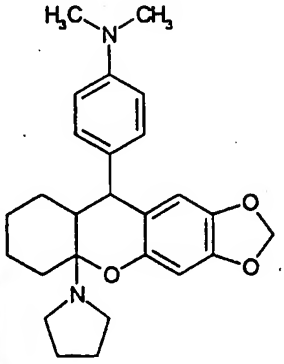
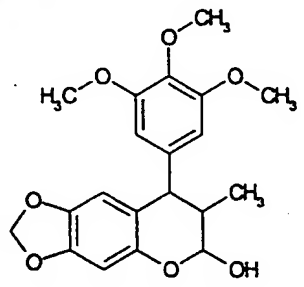
HA11-45	RCL R17,135-2	458.417	
HA11-46	2,6-DI-MEO-4-(7-ME-6-PYRROLIDIN-1-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	413.467	
HA11-47	RCL R17,138-7	420.55	
HA11-48	7-ME-8-(3,4,5-TRIMETHOXY-PHENYL)-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	374.387	

Table I Compounds for formula I

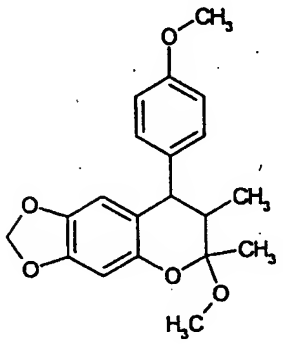
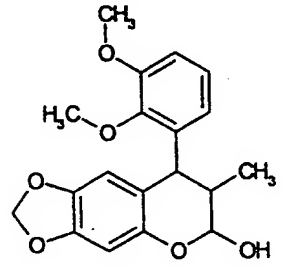
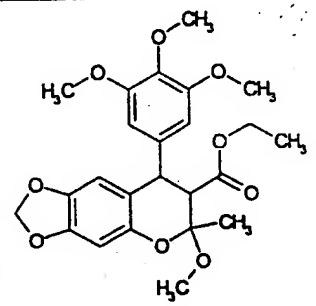
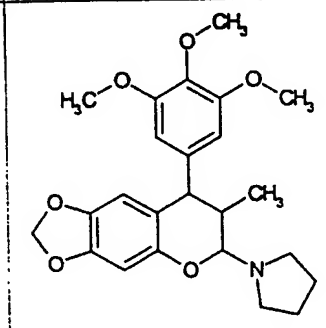
HA11-49	6-MEO-8-(4-MEO-PHENYL)-6,7-DIMETHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	342.389	
HA11-50	8-(2,3-DIMETHOXY-PHENYL)-7-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	344.361	
HA11-51	RCL R17,150-6	460.476	
HA11-52	1-[7-ME-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	427.494	

Table I Compounds for formula I

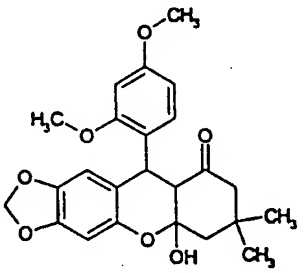
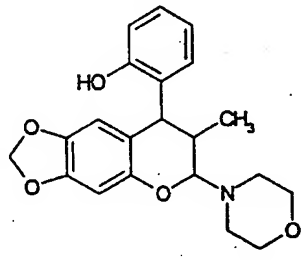
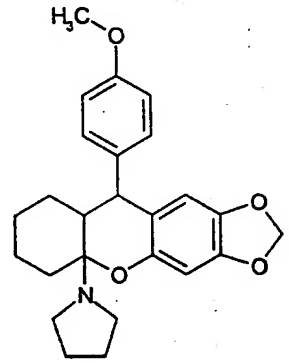
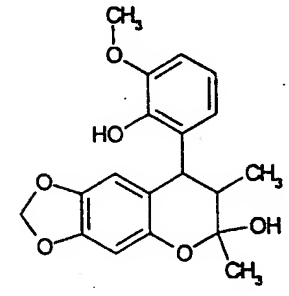
HA11-53	RCL R17,155-7	426.462	
HA11-54	2-(7-ME-6-MORPHOLIN-4-YL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	369.415	
HA11-55	RCL.R17,160-3	407.507	
HA11-56	8-(2-HO-3-MEO-PH)-6,7-DIMETHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	344.361	

Table I Compounds for formula I

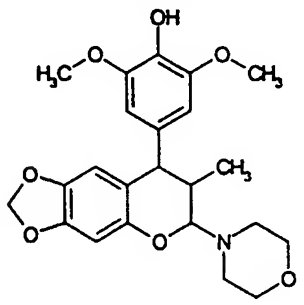
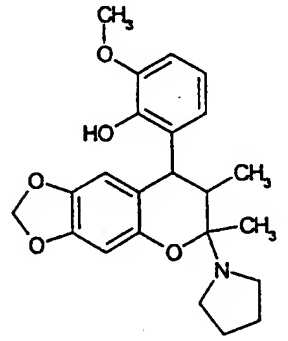
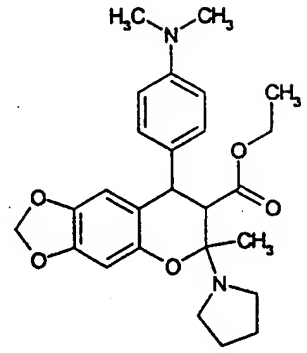
HA11-57	2,6-DI-MEO-4-(7-ME-6-MORPHOLIN-4-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	429.466	
HA11-58	2-(6,7-DI-ME-6-PYRROLIDIN-1-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-6-MEO-PHENOL	397.468	
HA11-59	RCL R17,171-9	452.548	

Table I Compounds for formula I

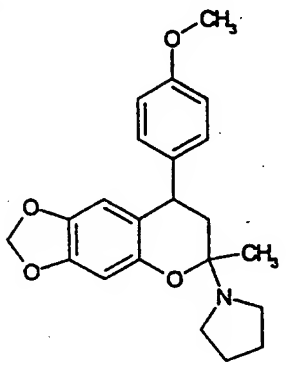
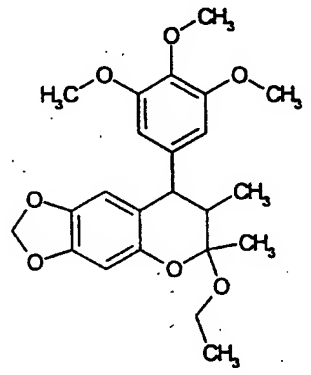
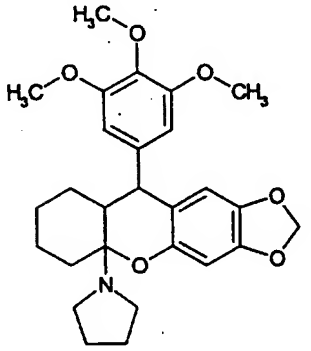
HA11-60	1-[8-(4-MEO-PH)-6-ME-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	367.443	
HA11-61	6-ETHOXY-6,7-DIMETHYL-8-(3,4,5-TRI-MEO-PH)-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	416.467	
HA11-62	RCL R17,174-3	467.559	

Table I Compounds for formula I

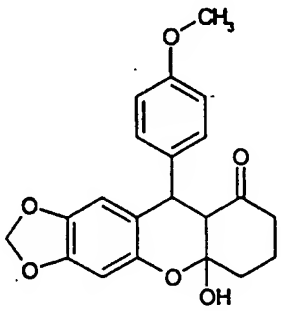
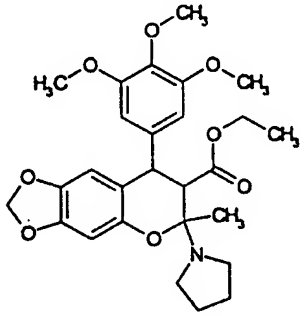
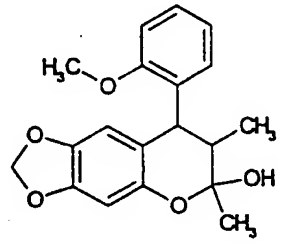
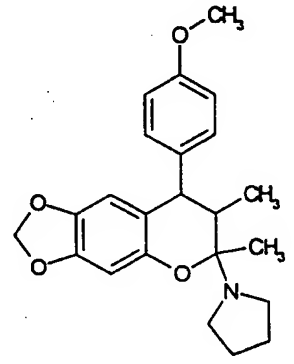
HA11-63	5A-HO-10-(4-MEO-PH)-HEXAHYDRO-1,3,5-TRIOXA-CYCLOPENTA[B]ANTHRACEN-9-ONE	368.383	
HA11-64	RCL R17,178-6	499.557	
HA11-65	8-(2-METHOXY-PHENYL)-6,7-DIMETHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	328.362	
HA11-66	1-[8-(4-MEO-PH)-6,7-DI-ME-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	381.469	

Table I Compounds for formula I

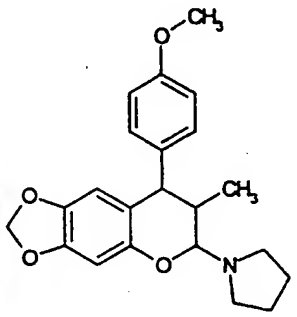
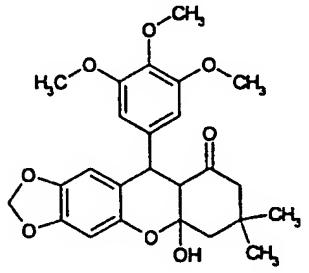
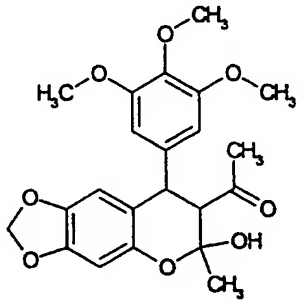
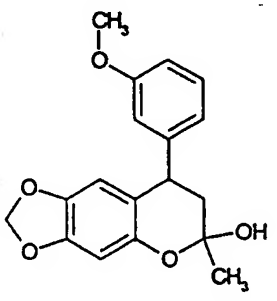
HA11-67	1-[8-(4-MEO-PH)-7-ME-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	367.443	
HA11-68	RCL R17,204-9	456.488	
HA11-69	1-[HO-6-ME-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-7-YL]-ETHANONE	416.424	
HA11-70	8-(3-METHOXY-PHENYL)-6-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	314.335	

Table I Compounds for formula I

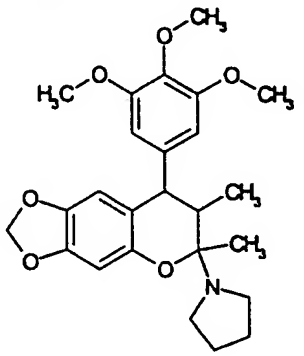
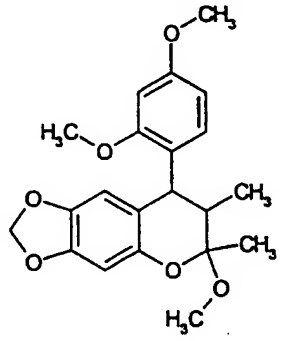
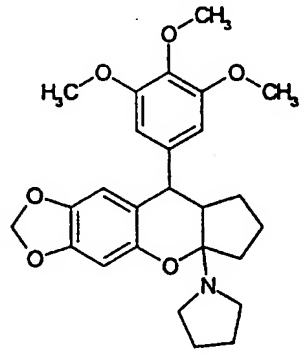
HA11-71	1-[6,7-DI-ME-8-(3,4,5-TRI-MEO-PH)-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-YL]-PYRROLIDINE	441.521	
HA11-72	8-(2,4-DIMETHOXY-PH)-6-MEO-6,7-DIMETHYL-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	372.415	
HA11-73	RCL R17,216-2	453.532	

Table I Compounds for formula I

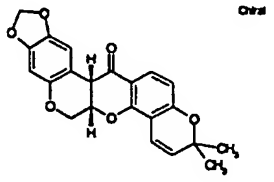
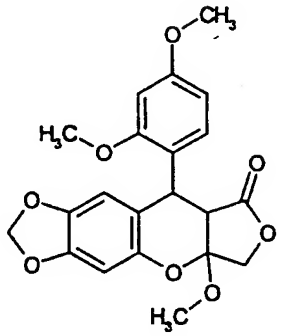
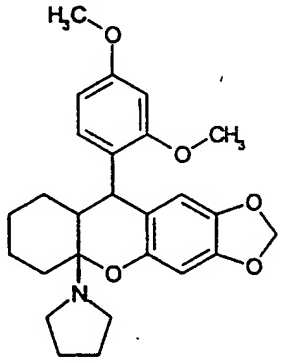
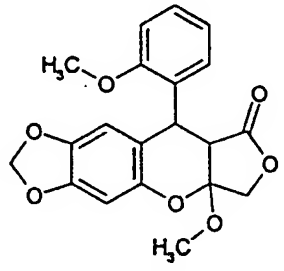
Comp. Number	MOLNAME	M.W	MOLSTRUCTURE
HA11-1	MILLETTONE	378.378	
HA11-2	9-(2,4-DI-MEO-PH)-5A-MEO-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA(B,G)NAPHTHALEN-8-ONE	400.381	
HA11-3	1-(10-(2,4-DIMETHOXY-PH)-1,3,5-TRIOXA-CYCLOPENTA(B)ANTHRACEN-5A-YL)-PYRROLIDINE	437.533	
HA11-4	5A-MEO-9-(2-MEO-PH)-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA(B,G)NAPHTHALEN-8-ONE	370.355	

Table I Compounds for formula I

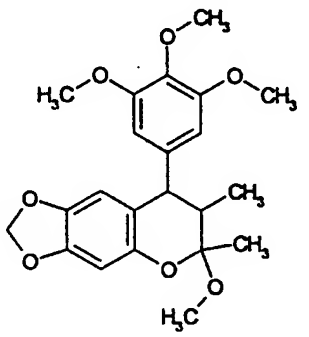
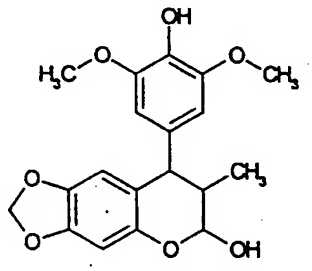
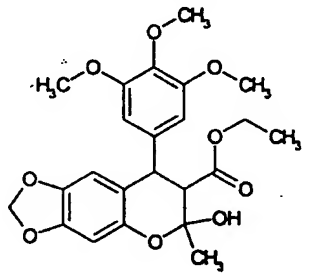
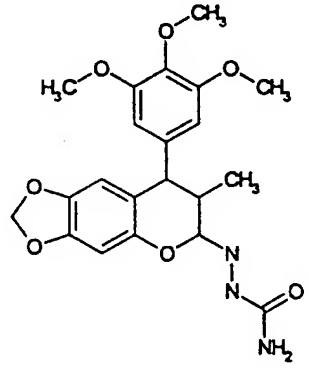
HA11-5	6-MEO-6,7-DIMETHYL-8-(3,4,5-TRIMETHOXY-PH)-7,8-2H-6H-(1,3)DIOXOLO(4,5-G)CHROMENE	402.44	
HA11-6	8-(4-HO-3,5-DIMETHOXY-PH)-7-ME-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	360.36	
HA11-7	6-HO-6-ME-8-(TRI-MEO-PH)-[1,3]DIOXOLO[4,5-G]CHROMENE-7-CARBOXYLIC ACID ET ESTER	446.449	
HA11-8	RCL R17,027-5	431.443	

Table I Compounds for formula I

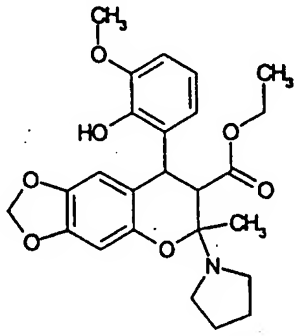
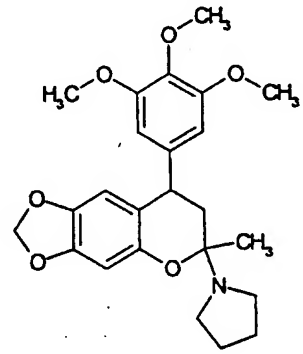
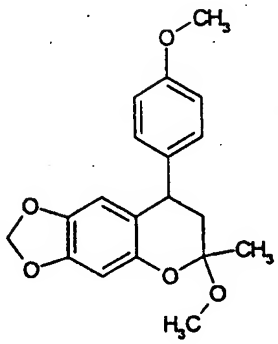
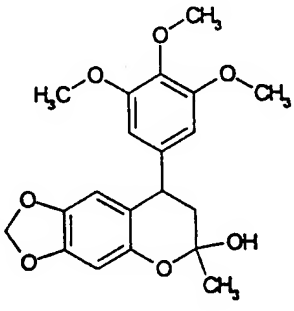
HA11-9	RCL R17,028-3	455.504	
HA11-10	1-[6-ME-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	427.494	
HA11-11	6-MEO-8-(4-METHOXY-PHENYL)-6-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	328.362	
HA11-12	6-ME-8-(3,4,5-TRIMETHOXY-PHENYL)-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	374.387	

Table I Compounds for formula I

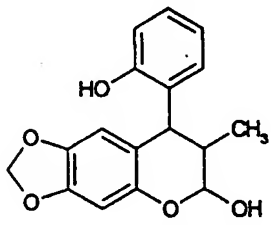
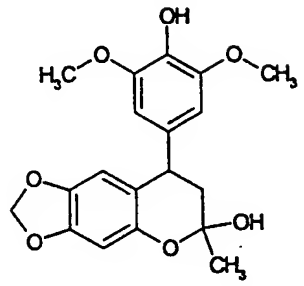
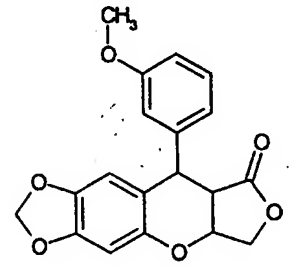
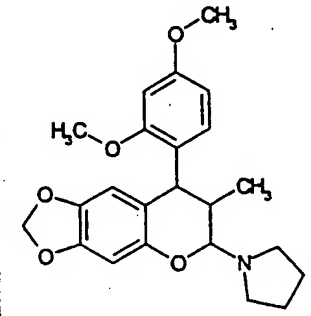
HA11-13	8-(2-HYDROXY-PHENYL)-7-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	300.308	
HA11-14	8-(4-HO-3,5-DIMETHOXY-PH)-6-ME-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	360.36	
HA11-15	9-(3-MEO-PH)-5A,6,8A,9-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA[B,G]NAPHTHALEN-8-ONE	340.329	
HA11-16	1-[8-(2,4-DI-MEO-PH)-7-ME-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	397.468	

Table I Compounds for formula I

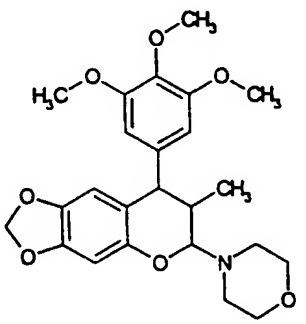
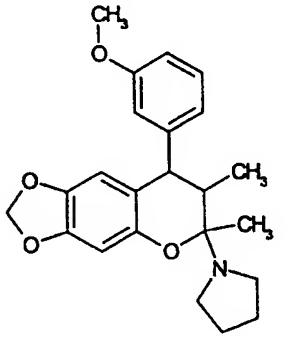
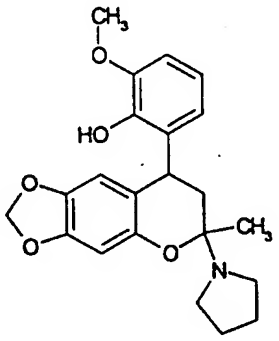
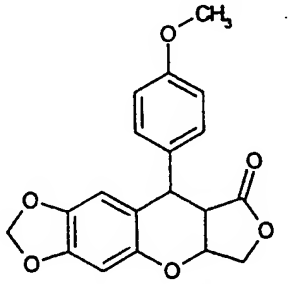
HA11-17	4-[7-ME-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-MORPHOLINE	443.493	
HA11-18	1-[8-(3-MEO-PH)-6,7-DI-ME-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	381.469	
HA11-19	2-MEO-6-(6-ME-6-PYRROLIDIN-1-YL-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	383.441	
HA11-20	9-(4-MEO-PH)-5A,6,8A,9-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA[B,G]NAPHTHALEN-8-ONE	340.329	

Table I Compounds for formula I

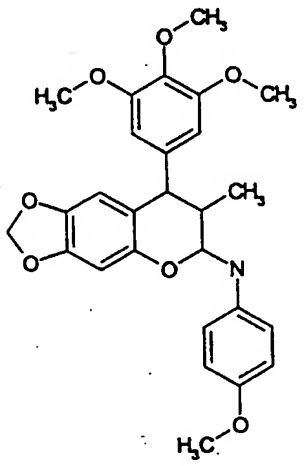
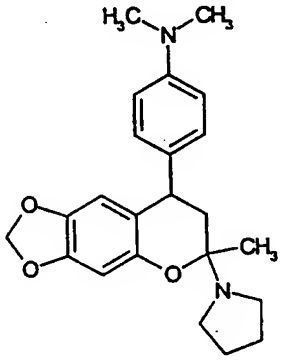
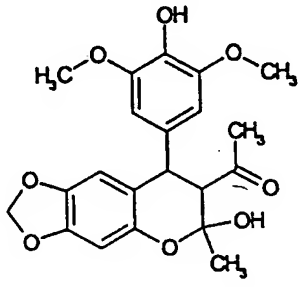
HA11-21	(4-MEO-PH)-[7-ME-8-(3,4,5-TRI-MEO-PH)-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-AMINE	479.526	
HA11-22	DI-ME-[4-(6-ME-6-PYRROLIDIN-1-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PH]-AMINE	380.485	
HA11-23	1-[6-HO-8-(4-HO-3,5-DI-MEO-PH)-6-ME-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-7-YL]-ETHANONE	402.397	

Table I Compounds for formula I

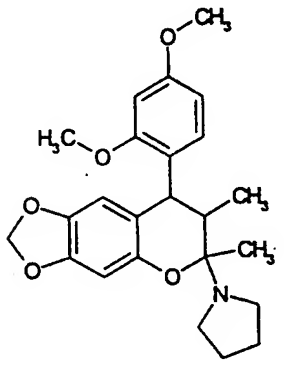
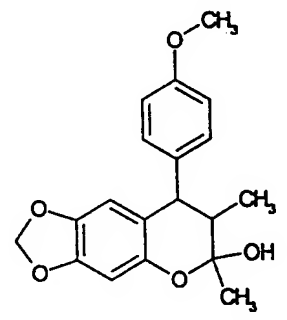
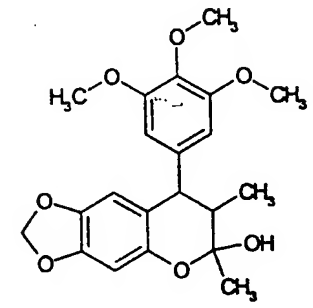
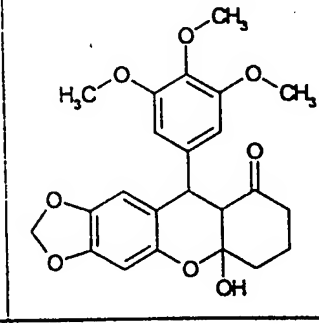
HA11-24	1-[8-(2,4-DI-MEO-PH)-6,7-DI-ME-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	411.495	
HA11-25	8-(4-METHOXY-PHENYL)-6,7-DIMETHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	328.362	
HA11-26	6,7-DIMETHYL-8-(3,4,5-TRIMETHOXY-PH)-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	388.414	
HA11-27	5A-HO-10-(3,4,5-TRI-MEO-PH)-HEXAHYDRO-1,3,5-TRIOXA-CYCLOPENTA[B]ANTHRACEN-9-ONE	428.435	

Table I Compounds for formula I

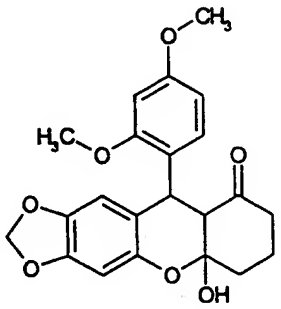
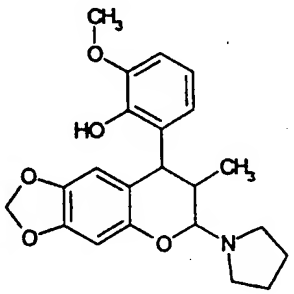
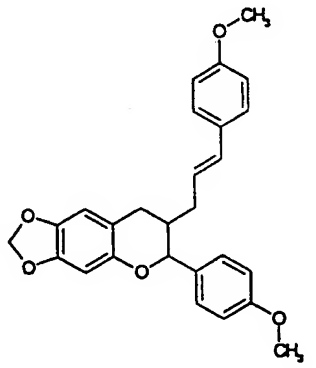
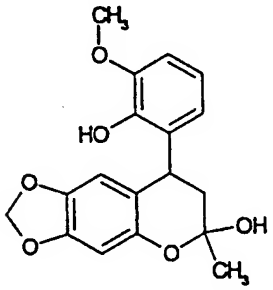
HA11-28	10-(2,4-DIMETHOXY-PH)-5A-HO-HEXAHYDRO-1,3,5-TRIOXA-CYCLOPENTA[B]ANTHRACEN-9-ONE	398.409	
HA11-29	2-MEO-6-(7-ME-6-PYRROLIDIN-1-YL-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	383.441	
HA11-30	6-(4-MEO-PH)-7-[3-(4-MEO-PH)-ALLYL]-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	430.497	
HA11-31	8-(2-HO-3-MEO-PHENYL)-6-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	330.334	

Table I Compounds for formula I

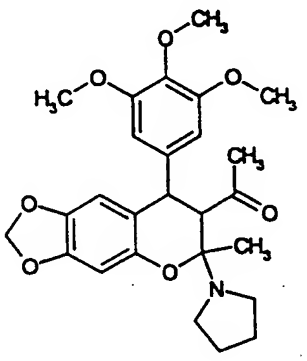
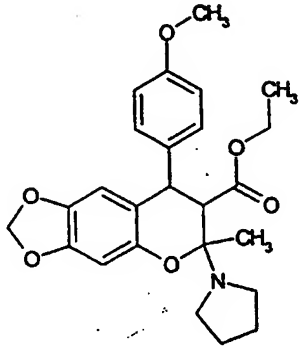
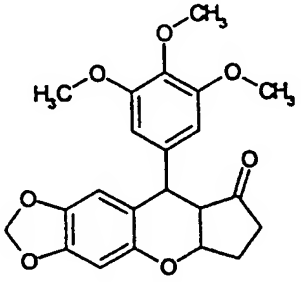
HA11-32	RCL R17,093-3	469.531	
HA11-33	RCL R17,094-1	439.505	
HA11-34	9-(3,4,5-TRI-MEO-PH)-4H-5AH-1,3,5-TRIOXA-DICYCLOPENTA[B,G]NAPHTHALEN-8-ONE	398.409	

Table I Compounds for formula I

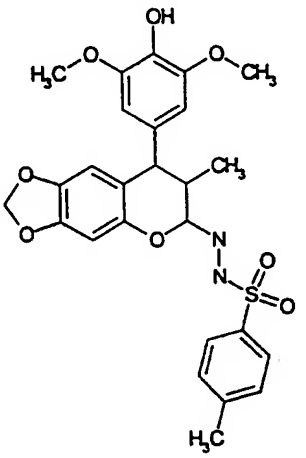
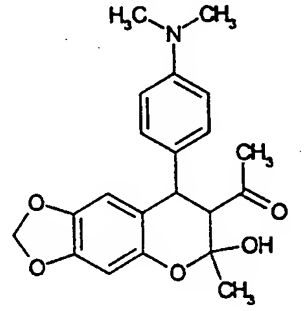
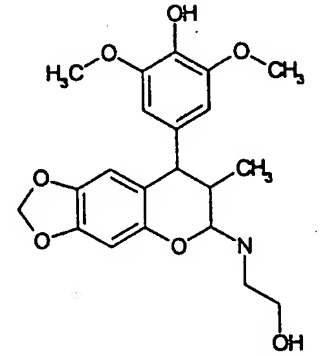
HA11-35	RCL R17,0976	528.579	
HA11-36	1-[8-(4-DI-ME-AMINO-PH)-6-HO-6-ME-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-7-YL]-ETHANONE	369.415	
HA11-37	RCL R17,106-9	403.428	

Table I Compounds for formula I

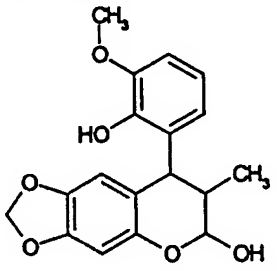
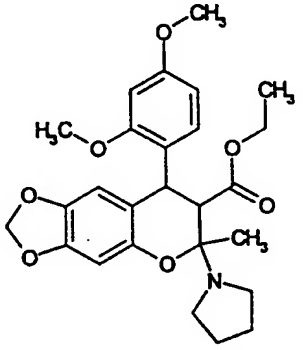
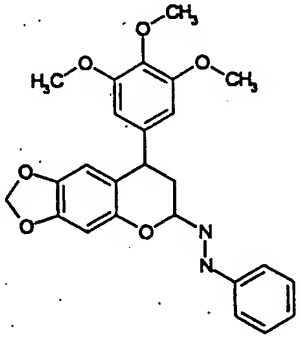
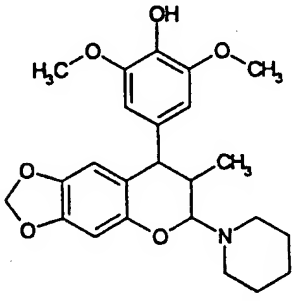
HA11-38	8-(2-HO-3-MEO-PHENYL)-7-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	330.334	
HA11-39	RCL R17,118-2	469.531	
HA11-40	N-PH-N'-[8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-HYDRAZINE	450.488	
HA11-41	2,6-DI-MEO-4-(7-ME-6-PIPERIDIN-1-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	427.494	

Table I Compounds for formula I

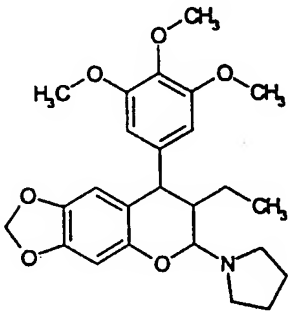
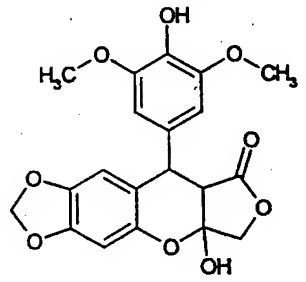
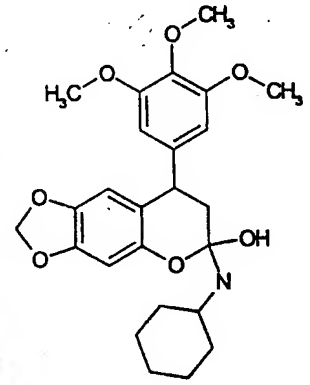
HA11-42	1-[7-ET-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	441.521	
HA11-43	5A-HO-9-(HO-3,5-DI-MEO-PH)-4H-1,3,5,7-TETRAOXA-DICYCLOPENTA[B,G]NAPHTHALEN-8-ONE	402.353	
HA11-44	6-CYCLOHEXYLAMINO-8-(3,4,5-TRI-MEO-PH)-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	457.52	

Table I Compounds for formula I

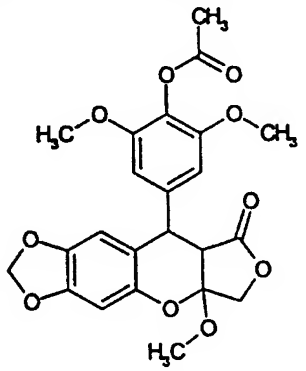
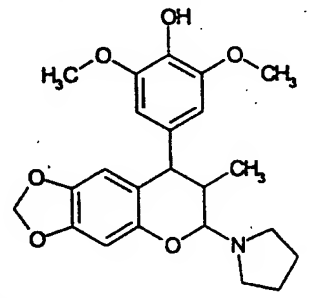
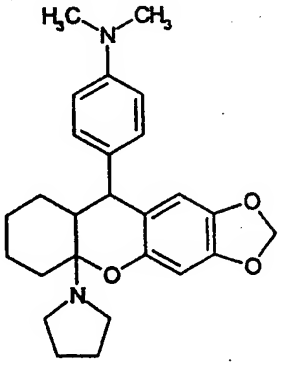
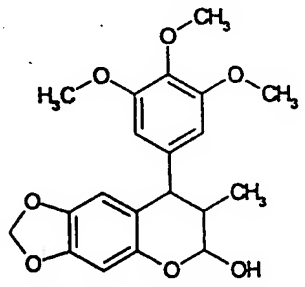
HA11-45	RCL R17,135-2	458.417	
HA11-46	2,6-DI-MEO-4-(7-ME-6-PYRROLIDIN-1-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	413.467	
HA11-47	RCL R17,138-7	420.55	
HA11-48	7-ME-8-(3,4,5-TRIMETHOXY-PHENYL)-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	374.387	

Table I Compounds for formula I

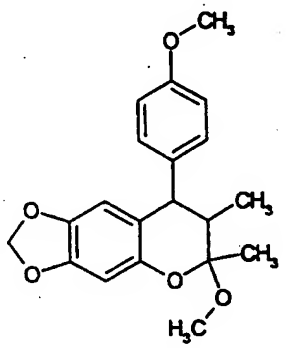
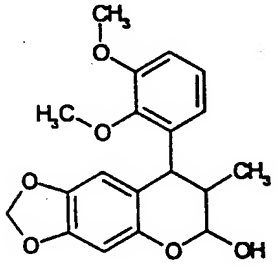
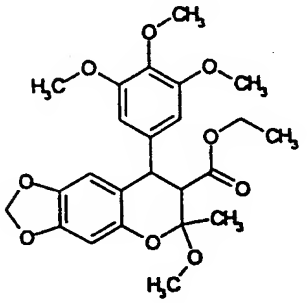
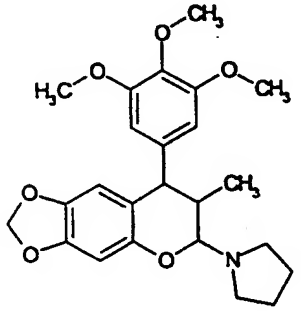
HA11-49	6-MEO-8-(4-MEO-PHENYL)-6,7-DIMETHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	342.389	
HA11-50	8-(2,3-DIMETHOXY-PHENYL)-7-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	344.361	
HA11-51	RCL R17,150-6	460.476	
HA11-52	1-[7-ME-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	427.494	

Table I Compounds for formula I

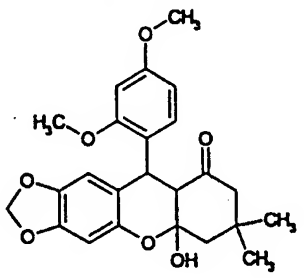
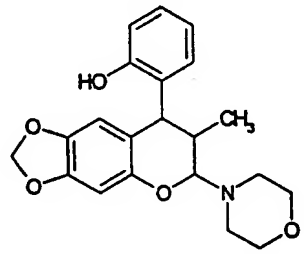
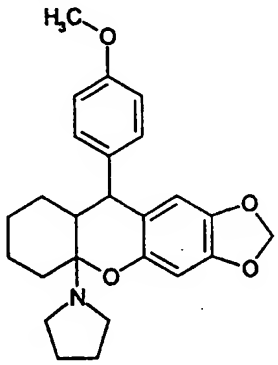
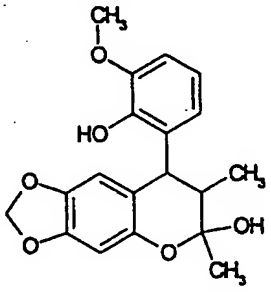
HA11-53	RCL R17,155-7	426.462	
HA11-54	2-(7-ME-6-MORPHOLIN-4-YL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	369.415	
HA11-55	RCL R17,160-3	407.507	
HA11-56	8-(2-HO-3-MEO-PH)-6,7-DIMETHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	344.361	

Table I Compounds for formula I

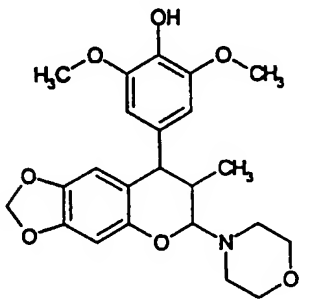
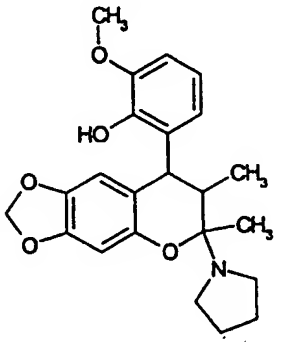
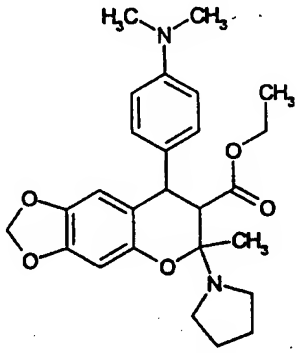
HA11-57	2,6-DI-MEO-4-(7-ME-6-MORPHOLIN-4-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-PHENOL	429.466	
HA11-58	2-(6,7-DI-ME-6-PYRROLIDIN-1-YL-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-8-YL)-6-MEO-PHENOL	397.468	
HA11-59	RCL R17,171-9	452.548	

Table I Compounds for formula I

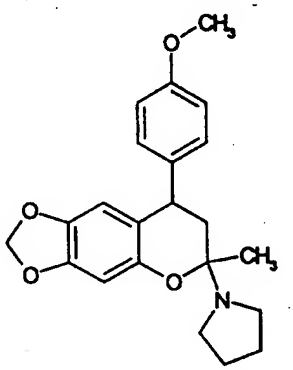
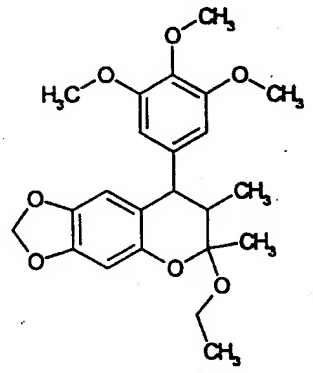
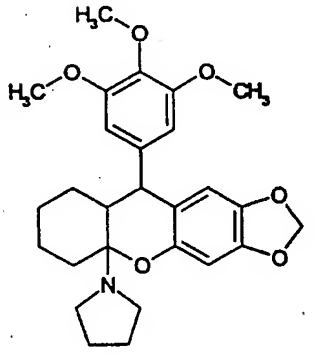
HA11-60	1-[8-(4-MEO-PH)-6-ME-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	367.443	
HA11-61	6-ETHOXY-6,7-DIMETHYL-8-(3,4,5-TRI-MEO-PH)-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	416.467	
HA11-62	RCL R17,174-3	467.559	

Table I Compounds for formula I

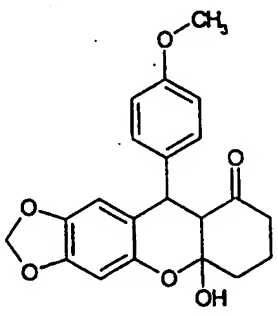
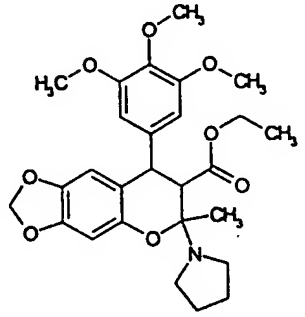
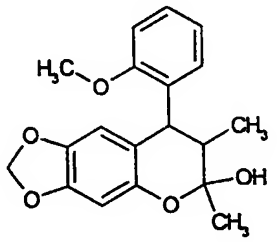
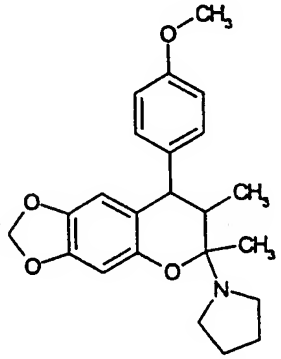
HA11-63	5A-HO-10-(4-MEO-PH)-HEXAHYDRO-1,3,5-TRIOXA-CYCLOPENTA[B]ANTHRACEN-9-ONE	368.383	
HA11-64	RCL R17,178-6	499.557	
HA11-65	8-(2-METHOXY-PHENYL)-6,7-DIMETHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	328.362	
HA11-66	1-[8-(4-MEO-PH)-6,7-DI-ME-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	381.469	

Table I Compounds for formula I

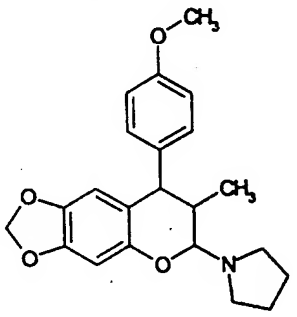
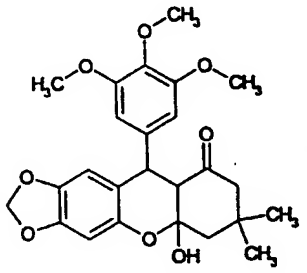
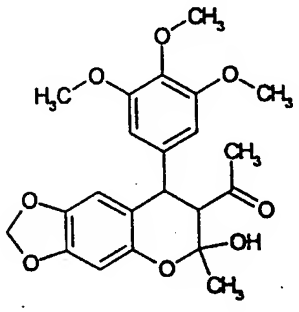
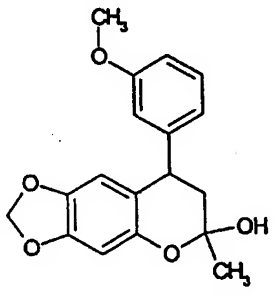
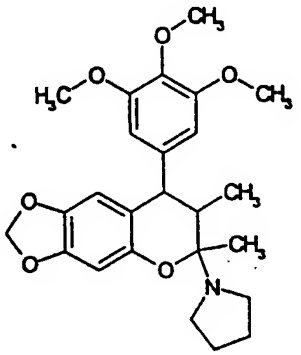
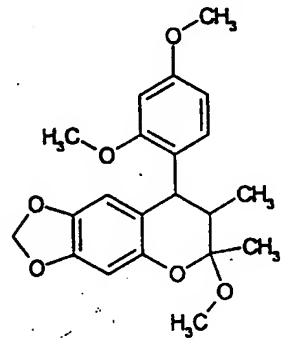
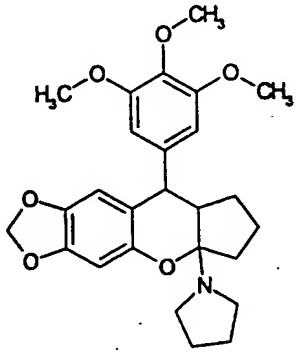
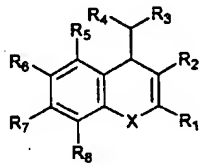
HA11-67	1-[8-(4-MEO-PH)-7-ME-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-YL]-PYRROLIDINE	367.443	
HA11-68	RCL R17,204-9	456.488	
HA11-69	1-[HO-6-ME-8-(3,4,5-TRI-MEO-PH)-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-7-YL]-ETHANONE	416.424	
HA11-70	8-(3-METHOXY-PHENYL)-6-METHYL-7,8-DIHYDRO-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-6-OL	314.335	

Table I Compounds for formula I

HA11-71	1-[6,7-DI-ME-8-(3,4,5-TRI-MEO-PH)-6H-[1,3]DIOXOLO[4,5-G]CHROMEN-YL]-PYRROLIDINE	441.521	
HA11-72	8-(2,4-DIMETHOXY-PH)-6-MEO-6,7-DIMETHYL-7,8-2H-6H-[1,3]DIOXOLO[4,5-G]CHROMENE	372.415	
HA11-73	RCL R17,216-2	453.532	

Preferred compounds according to formula III



III

include the compounds of Table II:

Table II **Compounds for formula III**

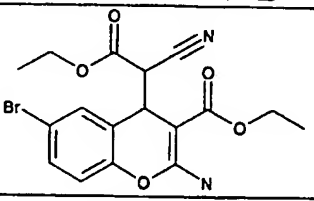
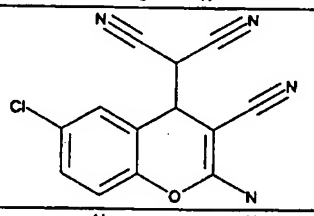
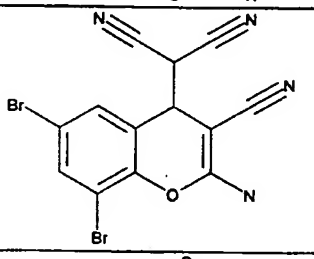
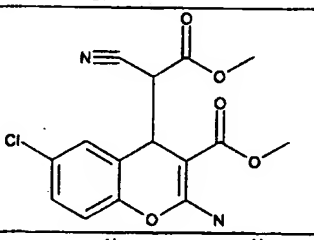
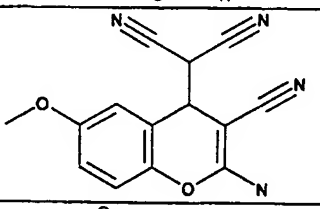
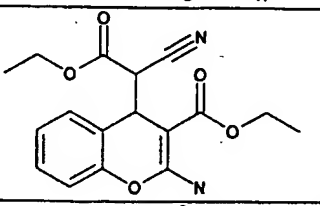
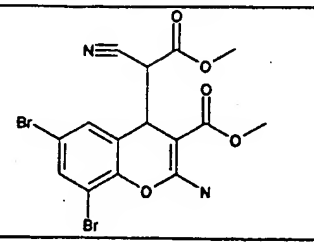
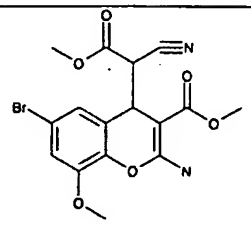
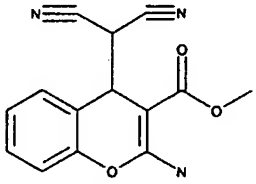
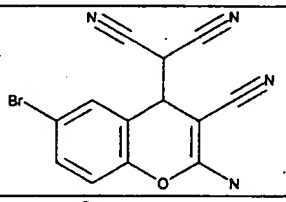
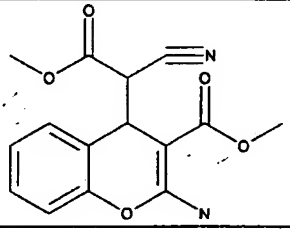
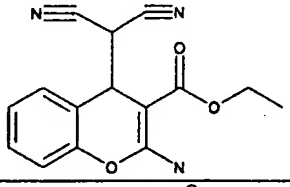
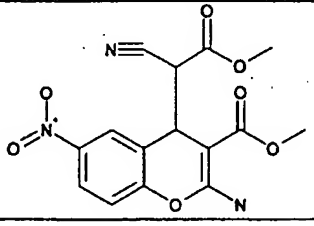
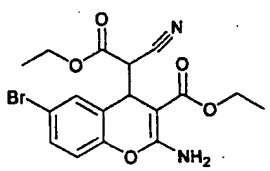
Comp. Number	MOLNAME	M.W.	MOLSTRUCTURE
HA14-1	ETHYL 2-AMINO-6-BROMO-4-(1-CYANO-2-ETHOXY-2-OXOETHYL)-4H-CHROMENE-3-CARBOXYLATE	409.2340	
HA14-2	2-(2-AMINO-6-CHLORO-3-CYANO-4H-CHROMEN-4-YL)MALONONITRILE	270.6780	
HA14-3	2-(2-AMINO-6,8-DIBROMO-3-CYANO-4H-CHROMEN-4-YL)MALONONITRILE	394.0250	
HA14-4	METHYL 2-AMINO-6-CHLORO-4-(1-CYANO-2-METHOXY-2-OXOETHYL)-4H-CHROMENE-3-CARBOXYLA	336.7300	
HA14-5	2-(2-AMINO-3-CYANO-6-METHOXY-4H-CHROMEN-4-YL)MALONONITRILE	266.2590	
HA14-6	ETHYL 2-AMINO-4-(1-CYANO-2-ETHOXY-2-OXOETHYL)-4H-CHROMENE-3-CARBOXYLATE	330.3380	
HA14-7	METHYL 2-AMINO-6,8-DIBROMO-4-(1-CYANO-2-METHOXY-2-OXOETHYL)-4H-CHROMENE-3-CARBOX	460.0770	

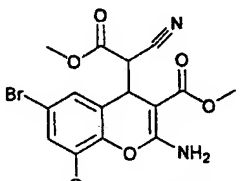
Table II Compounds for formula III

HA14-8	METHYL 2-AMINO-6-BROMO-4-(1-CYANO-2-METHOXY-2-OXOETHYL)-8-METHOXY-4H-CHROMENE-3-	411.2070	
HA14-9	METHYL 2-AMINO-4-(DICYANOMETHYL)-4H-CHROMENE-3-CARBOXYLATE	269.2590	
HA14-10	2-(2-AMINO-6-BROMO-3-CYANO-4H-CHROMEN-4-YL)MALONONITRILE	315.1290	
HA14-11	METHYL 2-AMINO-4-(1-CYANO-2-METHOXY-2-OXOETHYL)-4H-CHROMENE-3-CARBOXYLATE	302.2850	
HA14-12	ETHYL 2-AMINO-4-(DICYANOMETHYL)-4H-CHROMENE-3-CARBOXYLATE	283.2860	
HA14-13	METHYL 2-AMINO-4-(1-CYANO-2-METHOXY-2-OXOETHYL)-6-NITRO-4H-CHROMENE-3-CARBOXYLAT	347.2820	

The compounds of Table II are available from Maybridge Chemical Company. Particularly preferred compounds of formula III include compounds HA14-1 and HA14-8:



HA14-1



HA14-8

Bcl-2 Binding Assay for Candidate Inhibitors of Bcl-2

To measure the specific Bcl-2 binding of computer-predicted organic inhibitors, a Bcl-2 competition binding assay may be employed. The assay is based on fluorescence polarization. The assay can rapidly measure Bcl-2 receptor-ligand interaction without using filter binding, electrophoresis, or precipitation steps. Fluorescence polarization gives a direct, instantaneous equilibrium measure of the bound/free ratio between ligand and receptor molecules.

In order to set up the competition binding assay, the specific binding of a known peptide ligand of the targeted Bcl-2 functional pocket was first demonstrated. The peptide, designated peptide 1193 (GQVGRQLAIIGDDINR), is derived from the BH3 domain of the death agonist Bak. It has been shown in high-resolution X-ray structure to bind strongly to the Bcl-2 pocket (Muchmore *et al.*, *Nature* 381:335-41, 1996; Sattler *et al.*, *Science* 275:983-6, 1997) Peptide 1193 was synthesized and labeled with a fluorescein tracer (Flu-1193). The binding affinity of Flu-1193 to the Bcl-2 protein (purified soluble Bcl-2 proteins purchased from Santa Cruz Biotechnology, Inc., CA) was determined by a saturation experiment. Since the polarization value is derived from the ratio of bound versus free tracer, the lowest concentration of Flu-1193 was chosen, such that the

concentration would yield a reasonable fluorescent signal and a stable polarization value. Using a fixed concentration of Flu-1193 peptide, Bcl-2 protein was titrated at increasing concentrations to achieve a saturated binding. The binding of the Flu-1193 peptide to Bcl-2 protein was measured on a LS-50 luminescence spectrometer equipped with polarizers using a dual path length quartz cell (500µL) (Perkin-Elmer Corp.). The fluorophore is excited with vertical polarized light at 485 nm (excitation slit width 10 nm), and the polarization value of the emitted light is observed through vertical and horizontal polarizers at 520 nm (emission slit width 10 nm).

Figure 1 illustrates a nonlinear least-squares fit for a saturation experiment using Flu-1193 and Bc1-2 protein in which the Bcl-2 concentration varied from 6nM to 2µM and Flu-1193 concentration remained at 30nM. The dissociation constant K_D of Flu-1193 was determined to be approximately 0.2 µM by using a nonlinear least-squares fit and single-site binding mode ($R^2 = 0.99$).

The binding affinity was also analyzed by Scatchard analysis. The Scatchard analysis is a standard method for analyzing the equilibrium binding parameters of a labeled molecule wily its target protein. The Scatchard plot is sensitive to presence of nonspecific binding, positive or negative cooperativity, and multiple classes of binding sites. The K_D calculated from the Scatchard plot (K_D= 1/slope), is approximately 0.25 µM which is in agreement with the value from dose-response calculation (K_D ~0.20µM). The data fit best to linear function, indicating a single class of binding site.

To further verify the specificity of the interaction of Flu-1193 and Bcl-2, a number of control experiments were carried out including measuring the binding of Flu-1193 to other proteins such as Bax, CD4, and the SH3 domain of the Bcr-Abl oncoprotein, and measuring the Bcl-2 binding of other Flu-labeled peptides derived from CD4 (Flu-1250 and Flu-1251) and Bcr-Abl SH3 (Flu-1217) (Fig. 2). The lack of binding interaction detected in these control systems (the signals were close to the background

level of free Flu-1193), demonstrated the specificity of the binding of Flu-1193 to Bcl-2.

Using Flu-1193 as a specific probe, a competition binding protocol may be set up for non-peptide organic ligands of Bcl-2. The competition format utilizes fixed concentrations of Flu-1193 and Bcl-2 proteins (30nM and 0.55μM, respectively), with increasing concentrations of organic compounds added to generate inhibition curves. The binding equation proposed by Weinhold *et al.*, *J. Am. Chem. Soc.* 114:9270-9275, 1992, is then used to derive the dissociation constant K_D of an inhibitor from its competition inhibition curve,

$$[Inhibitor] = \frac{K_D}{K_L}\left[[Bcl-2]x\left(\frac{A_B - A}{A - A_F}\right) - [Flu-1193]x\left(\frac{A_B - A}{A_B - A_F}\right)\right] - K_D$$

wherein [*Inhibitor*], [*Bcl-2*], and [*Flu-1193*] are the concentrations of inhibitor, Bcl-2 protein and Flu-1193 peptide, respectively; K_L is the dissociation constant of the Flu-1193 peptide; *A* is the observed fluorescence anisotropy, *A=2P/(3-P)*, where *P* is the observed fluorescence polarization values; and A_B and A_F are fluorescence anisotropy values when all of the Flu-1193 peptide is either bound to the Bcl-2 protein (A_B) or free in solution (A_F). The K_D value is adjusted by a factor of 5 as suggested by others for FP-based assays.

The dissociation constant of small molecule organic inhibitors of Bcl-2 identified in the foregoing binding assay with peptide Flu-1193 is preferably no more than about 500 μM, preferably no more than about 100 μM, most preferably no more than about 10 μM.

Biological Activity Assay of Candidate Inhibitors of Bcl-2

The small organic compounds which bind to the active pocket of Bcl-2 as described above may be tested for inhibition of Bcl-2 biological activity, and hence the ability to induce apoptosis in cancer cells where Bcl-2 proteins play a role in resisting apoptosis.

DNA fragmentation is an important and characteristic marker of apoptosis. Accordingly, cells of a variant of HL-60 are incubated with test compound at 100μM concentration for 24 hours. The DNA of the cells is then isolated by conventional techniques and analyzed for fragmentation on 2% agarose gels containing 0.2μg/ml ethidium bromide. Visible DNA fragmentation resulting from incubation of 100μM of compound with the cells for 24 hours generally indicates that the compound is active in inducing apoptosis.

Therapeutic Administration

The small molecule inhibitors of Bcl-2 function may be used to treat any condition characterized by the accumulation of cells which are regulated by Bcl-2. For the most part, the cells express or overexpress Bcl-2. Enhancement of Bcl-2 expression has been demonstrated to increase the resistance of cells to almost any apoptotic signal (Hockenbery *et al.*, *Nature* 348, 334 (1990); Nuñez *et al.*, *Immunol.* 144, 3602 (1990); Vaux *et al.*, *Nature* 335, 440 (1988); Hockenbery *et al.*, *Cell 75, 241 (1993); Ohmori et al., Res. Commun.* 192, 30 (1993); Lotem *et al.*, *Cell Growth.Differ* 4, 41 (1993); Miyashita *et al.*, *Blood* 81, 115 (1993); Minn et al.)). Principally, the proliferative disorders associated with the inhibition of cell apoptosis include cancer, autoimmune disorders and viral infections. Overexpression of Bcl-2 specifically prevents cells from initiating apoptosis in response to a number of stimuli (Hockenbery *et al.*, *Nature* 348, 334 (1990); Nunez *et al.*, *J. Immunol.* 144, 3602 (1990); Vaux *et al.*, *Nature* 335, 440 (1988); Hockenbery *et al.*, *Cell* 75, 241 (1993)). The induction of genes that inhibit Bcl-2 can induce apoptosis in a wide variety of tumor types, suggesting that many tumors continually rely on Bcl-2 or related gene products to prevent cell death. Bcl-2 expression has been associated with a poor prognosis in at least prostatic cancer, colon cancer and neuroblastoma (McDonnell *et al.*, *Cancer Res.* 52, 6940 (1992); Hague *et al.*, *Oncogene* 9, 3367 (1994); Castle *et al.*, *Am. J. Pathol.* 143, 1543 (1993)). Bcl-2 or the related gene

Bcl_x has been found to confer resistance to cell death in response to several chemotherapeutic agents (*Ohmori et al., Res. Commun.* 192, 30 (1993); Lotem *et al., Cell Growth.Differ* 4, 41 (1993); Miyashita *et al., Blood* 81, 115 (1993); Minn et al.)).

Physiologic cell death is important for the removal of potentially autoreactive lymphocytes during development and for the removal of excess cells after the completion of an immune response. Failure to remove these cells can result in autoimmune disease. A lupus-like autoimmune disease has been reported in transgenic mice constitutively overexpressing Bcl-2 in their B cells (Stressed *et al., Proc. Natl. Acad. Sci. USA* 88, 8661 (1991)). Linkage analysis has established an association between the Bcl-2 locus and autoimmune diabetes in non-obese diabetic mice (Garchon *et al., Eur. J. Immunol.* 24, 380 (1994). The compounds of the invention may be used to induce apoptosis of self-reactive lymphocytes. By "self-reactive" is meant a lymphocyte which participates in an immune response against antigens of host cells or host tissues.

The small molecule inhibitors of Bcl-2 function may be used in the treatment of viral infection, to induce apoptosis of virally infected cells. Viruses have developed mechanisms to circumvent the normal regulation of apoptosis in virus-infected cells, and theses mechanisms have implicated Bcl-2. For example, the E1B 19-kDa protein is instrumental in the establishment of effective adenoviral infection. The apoptosis-blocking ability of E1B can be replaced in adenoviruses by Bcl-2 (Boyd *et al., Cell* 79, 341 (1994)). Genes of certain other viruses have been shown to have sequence and functional homology to Bcl-2 (Neilan *et al., J. Virol.* 67, 4391 (1993); Henderson *et al., Proc. Natl. Acad. Sci. U.S.A.* 90, 8479 (1993)). The viral gene LMP-1 specifically upregulates Bcl-2 providing a survival advantage over latently infected cells (Henderson *et al., Cell* 65, 1107 (1991)). Sindbis infection is dependent on the host cell's expression of Bcl-2 (Levine *et al., Nature* 361,739 (1993)).

The effective amount of compound needed to treat a subject may be routinely determined through procedures well known to those skilled in the art which address such parameters as biological half-life, bioavailability, and toxicity. Such determination is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

The present invention provides pharmaceutical compositions that comprise the compounds of the invention and pharmaceutically acceptable carriers or diluents.

For parenteral administration, the compounds of the invention can be, for example, formulated as a solution, suspension, or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. The vehicle or lyophilized powder may contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). For example, a parenteral composition suitable for administration by injection is prepared by dissolving 1.5% by weight of active ingredient in 0.9% sodium chloride solution. The formulation can be sterilized by any commonly used technique.

The pharmaceutical compositions according to the invention may be administered as a single dose or in multiple doses. The pharmaceutical compositions of the present invention may be administered either as individual therapeutic agents or in combination with each other or with other therapeutic agents. The treatments of the present invention may be combined with conventional therapies, which may be administered sequentially or simultaneously.

The pharmaceutical compositions of the present invention may be administered by any means that enables the active agent to reach the targeted cells. Because compounds of the invention may be subject to being digested when administered orally, parenteral administration, i.e.,

intravenous, subcutaneous or intramuscular, would ordinarily be used to optimize absorption. Intravenous administration may be accomplished with the aid of an infusion pump. Alternatively, the compounds of the invention can be formulated as aerosol medicaments for intranasal inhalation or topical administration.

The dosage administered varies depending upon factors such as: pharmacodynamic characteristics; its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms; kind of concurrent treatment; and frequency of treatment. Usually, the dosage of the compound of the invention can be about 1 to 3000 milligrams per 50 kilograms of body weight; preferably 10 to 1000 milligrams per 50 kilograms of body weight; more preferably 25 to 80 milligrams per 50 kilograms of body weight. Ordinarily, 8 to 800 milligrams are administered to an individual per day in divided doses 1 to 6 times a day or in sustained release form is effective to obtain desired results.

Example 1

Bcl-2 Ligand Binding Assay

The binding of selected organic compounds to Bcl-2 protein in the presence of peptide Flu-1193 was measured on a LS-50 luminescence spectrometer equipped with polarizers using a dual path length quartz cell (500µL) (Perkin-Elmer Corp.). The fluorophore was excited with vertical polarized light at 485 nm (excitation slit width 10 nm). The polarization value of the emitted light was observed through vertical and horizontal polarizers at 520 nm (emission slit width 10 nm). Fixed concentrations of Flu-1193 and Bcl-2 proteins (30nM and 0.55µM, respectively), with increasing concentrations of test compound was added to generate inhibition curves. The binding equation proposed by Weinhold *et al.*, *J. Am. Chem. Soc.* 114:9270-9275, 1992, was used to derive the dissociation constant K_D of the test compound from its competition inhibition curve,

$$[Inhibitor] = \frac{K_D}{K_L}\left[[Bcl-2]x\left(\frac{A_B - A}{A - A_F}\right) - [Flu-1193]x\left(\frac{A_B - A}{A_B - A_F}\right)\right] - K_D$$

wherein [*Inhibitor*], [*Bcl-2*], and [*Flu-1193*] are the concentrations of inhibitor, Bcl-2 protein and Flu-1193 peptide, respectively; K_L is the dissociation constant of the Flu-1193 peptide; *A* is the observed fluorescence anisotropy, *A=2P/(3-P)*, where *P* is the observed fluorescence polarization values; and A_B and A_F are fluorescence anisotropy values when all of the Flu-1193 peptide is either bound to the Bcl-2 protein (A_B) or free in solution (A_F).

According to this binding protocol, 716 organic compounds selected from computer screening studies were initially tested at 100 μM concentration. A group of compounds found to be active in the assay with a level of inhibition ranging from 35% to 98%. Four of the active compounds comprised compounds HA12-16 (compound HA12-16 may also be identified herein as "HA01"), HA02, HA03 and HA04. A clear concentration-dependent competition binding was observed for these compounds and their biding affinities determined by the above procedure. The two most potent compounds, HA12-16 and HA02, exhibited a binding affinity (K_D) of 7 μM and 15 μM, respectively.

Compound HA14-1 was tested in the same manner. A clear concentration-dependent competition binding was observed for this compound over a concentration of from 1 to 100 μM. The results are set forth in Fig. 6.

Example 2

DNA Fragmentation Assay A

The cells for this assay comprised a variant of the human myeloid leukemia HL-60 cell line transfected with Bcl-2 to overexpress Bcl-2 (Liu *et al.*, *Cell* 86:147-57, 1996). Cells of the parent line are sensitive to 50 μM of the apoptosis-inducing drug etoposide. The Bcl-2-transfected line is

resistant to the same concentration of drug, indicating that Bcl-2 blocked apoptosis by the drug.

The Bcl-transfected cells were incubated with Bcl-2 inhibitor test compounds at 50 μM for 24 hours and then examined for apoptosis by the following DNA fragmentation assay. Then the treated cells were washed in PBS, lysed in digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH8, 25 mM EDTA, pH 8, 0.5% SDS, 0.1 mg/ml proteinase K), and incubated overnight at 50°C. The samples were extracted three times with phenol-chloroform, precipitated with an equal volume isopropanol, and spun down for 15 minutes in a microcentrifuge at room temperature. The DNA precipitate was washed once with 70% ethanol and resuspended in TE buffer containing 200 μg/ml DNase-free RNase A (Boehringer Mannheim, Indianapolis, IN). Alter incubation at 37°C for 30 min., the DNA was loaded into a 2% agarose mini-gel with 2 μg/ml ethidium bromide, and electrophoresis is run at 50 V for 2 hours in 0.5 x TBE buffer. The gel was destained with water for 1 hour arid photographed under UV light.

The results are shown in Fig. 3: lane 0, control; lane 1, compound HA13; lane 2, compound HA14; lane 3, compound HA11-57. DNA fragmentation is apparent in each lane, except for the control, indicating that each compound is effective in reversing Bcl-2 block of apoptosis.

Example 3

DNA Fragmentation Assay B

The assay of Example 2 was repeated for compounds HA01 (also designated "HA12-16"), HA02 and HA04, with the following modifications. First, the ethidium bromide stain concentration was 1 μg/ml instead of 2 μg/ml. Second, the cells for the assay comprised 697 cells. The 697 line is a human pre-B leukemia line with a t(1;19) chromosomal translocation (no translocation involving Bcl-2). Bcl-2 is highly expressed in this line. The high expression of Bcl-2 in 697 cells was confirmed by protein

immunoblot analysis (data not shown). Taxol (Sigma Chemical Co., St. Louis, MO), a widely-used anticancer drug known to induce apoptosis in 697 cells, was used as a positive control. A randomly selected organic compound which was inactive in the Bcl-2 binding assay was included in the assay as a negative control. DNA markers were phiX174 DNA with restriction endonuclease Hae III (Boehringer Mannheim, Indianapolis, IN). The cells were incubated with test compound at 50 μM concentration or Taxol at 5 μM concentration for 48 hours. The results are shown in Fig. 4. The test compounds induced DNA fragmentation to various extents while the control compound did not show any effect.

A control experiment was carried out to investigate the specificity of the apoptosis-inducing effect of the compounds in a human myeloid leukemia HL-60 neo line in which apoptosis is not regulated by Bcl-2. the lack of effect in inducing apoptosis of the HL-60 neo cells by a representative compound HA01 at the same concentration (50μM) which induced apoptosis in the 697 cells (data not shown) indicated the specificity of the compound for the Bcl-2 mediated apoptotic pathway.

Example 4

DNA Fragmentation Assay C

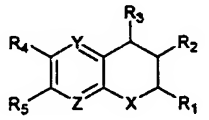
The assay of Example was repeated by incubating HL-60 Bcl-2 cells with compound HA14-1 (50 μM for 24 hours). Alternatively, the cells were first pretreated with 100 μM fluoromethyl ketone at 100 μM for 2 hours, followed by 50 μM HA14-1 for 24 hours. The results are set forth in Fig. 5: lane A, HA14-1; lane B, fluoromethyl ketone and HA14-1. Fluoromethyl ketone is an inhibitor of a downstream target of Bcl-2. Fluoromethyl ketone pretreatment of HL-60 Bcl-2 cells should neutralize the action of HA14-1. This is indeed shown in Fig. 5, lane B.

All references discussed herein are incorporated by reference. One skilled in the art will readily appreciate that the present invention is well

adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

CLAIMS

1. A method of inducing apoptosis of cells in a subject, which cells are regulated by Bcl-2, comprising administering to the subject an effective amount of an active compound of the formula I:



I

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; O; and S;

Y and Z are independently selected from the group consisting of CH and N; and when Z is N, then Y may further be $-CR_6$, where R_6 is selected from the group consisting of CH_3; OCH_3; CNH_2; and COH;

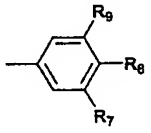
R_1 is selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; $NH(C_{1-3}$ alkyl); $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$;phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings;

R_2 is selected from the group consisting of hydrogen; C_{1-3} alkyl; C_{1-3} alkoxy; halogen; CF_3; NH_2; OH; COOH; $COOCH_3$; $CONH_2$; and $CONHCH_3$;

or, R_1 and R_2 together may form the group -$CH_2CH_2CH_2$- or -$CH_2CH_2CH_2CH_2$-;

or, R_1 and R_2 together may form, starting from R_1, the group -$NHCH_2CH_2$-, -$NHCOCH_2$-, or -$OCOCH_2$-;

R_3 is selected from the group consisting of hydrogen; CH_3; CF_3; OCH_3; NH_2; OH; COOH; $COCH_3$; $CH{=}CH_2$; $CH_2{=}CHCH_2$; $CH(CH_3)_2$; CH_2OH; CH_2NH_2; CH_2COOH; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, OCH_3 or CF_3; five- and six-member heterocyclic rings; and a substituted phenyl group of the formula:



wherein

R_7, R_8 and R_9 are independently selected from the group consisting of hydrogen, CH_3, CF_3, OH, OCH_3, CH_2OH and CHO; provided that at least two of the members of the group R_7, R_8 and R_9 must be OH or OCH_3 when the remaining member of the group is hydrogen, CH_3 or CF_3;

R_4 and R_5 are independently selected from the group consisting of hydrogen, CH_3, and OCH_3; and when Y and Z are both CH, R_4 and R_5 may be further selected from OH and NH_2;

or, R_4 and R_5 together may form the group -$CH_2CH_2CH_2$- or -$CH_2CH_2CH_2CH_2$-;

or, R_4 and R_5 together may form, starting from R_4, the group -$NHCH_2CH_2$-, -$NHCOCH_2$-, -$OCOCH_2$- or -$O(CH_2)_nO$-, wherein n is 1, 2 or 3;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

2. The method according to claim 1 wherein the compound has a dissociation constant of not more than about 500 μM for binding the hydrophobic pocket on the Bcl-2 protein formed by the BH1, BH2, and BH3 domains of the Bcl-2 protein.

3. The method according to claim 1 wherein R_1 is a heterocyclic ring selected from the group consisting of piperidino, piperazino, morpholino, pyrimidyl, pyrrolidino and imidazo.

4. The method according to claim 1 wherein R_3 is a heterocyclic ring selected from the group consisting of piperidinyl, piperazinyl, morpholino, pyrimidyl, pyrrolyl, pyrrolidino, and imidazyl.

5. The method according to claim 1 wherein R_7, R_8 and R_9 are all OCH_3.

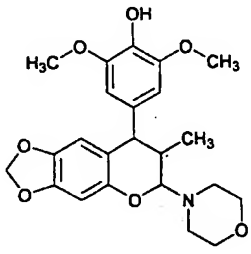
6. The method according to claim 1 wherein R_7 and R_9 are OCH_3, and R_8 is OH.

7. The method according to claim 1 wherein R_1 or R_3 is mono-substituted cyclohexyl, and the position of the substitution is *para*.

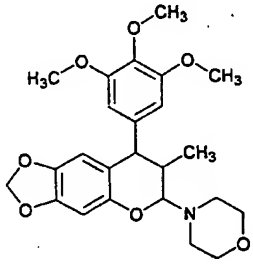
8. The method according to claim 1 wherein R_1 is mono-substituted phenyl, and the position of the substitution is *para*.

9. The method according to claim 7 wherein R_5 is CH_3, CH_2CH_3, COOH, $COCH_3$, $CONH_2$ or $CONHCH_3$.

10. The method according to claim 1 wherein the compound is selected from the group consisting of compound HA11-57 and compound HA11-17:



HA11-57



HA11-17

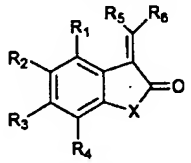
11. The method according to claim 1 wherein the compound causes the fragmentation of DNA in a Bcl-2 transfected HL-60 cell line when incubated with such cells at a concentration of not more than 100 μM for 24 hours.

12. The method according to claim 1 wherein the cells induced to undergo apoptosis comprise cancer cells.

13. The method according to claim 1 wherein the cells induced to undergo apoptosis comprise virus-infected cells.

14. The method according to claim 1 wherein the cells induced to undergo apoptosis comprise self-reactive lymphocytes.

15. A method of inducing apoptosis of cells in a subject which are regulated by Bcl-2 comprising administering to the subject an effective amount of an active compound of the formula II



II

wherein

R_1, R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; and $NH(C_{1-3}$ alkyl); and one of R_1, R_2, R_3 and R_4 may be phenyl or a heterocyclic ring; provided at least one of R_1, R_2, R_3 and R_4 must be hydrogen;

R_5 and R_6 are independently selected from the group consisting of hydrogen; CN; CH_2CN; $COOCH_3$; $CONH_2$; phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CH_3, OCH_3, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings; provided, only one of R_5 or R_6 may be phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic in the same compound, and further provided that when one of R_5 or R_6 is phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic, then the other must be hydrogen;

or at least one of R_5 and R_6 may be halogen, provided that the other must be C_{1-5} alkyl or C_{1-5} alkoxy.

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

16. The method according to claim 15 wherein the compound has a dissociation constant of not more than about 500 μM for binding the hydrophobic pocket on the Bcl-2 protein formed by the BH1, BH2, and BH3 domains of the Bcl-2 protein.

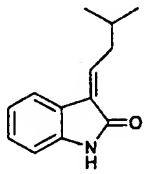
17. The method of claim 15 wherein at least one of R_1, R_2, R_3 and R_4 is selected from the group consisting of piperidino, piperazino, morpholino, pyrimidyl, pyrrolidino and imidazo.

18. The method of claim 15 wherein at least one of R_5 or R_6 is selected from the group consisting of pyrrolyl, imidazolyl, piperidinyl, piperazinyl, morpholino, pyrimidyl and pyrrolidino.

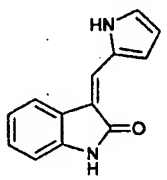
19. The method of claim 15 wherein one of R_5 or R_6 is substituted phenyl.

20. The method of claim 15 wherein one of R_5 or R_6 is mono-substituted phenyl or mono-substituted cyclohexyl, and the position of the substitution is *para*.

21. The method according to claim 15 wherein the compound is selected from the group consisting of compounds HA12-3 and HA12-16:



HA12-3



HA12-16

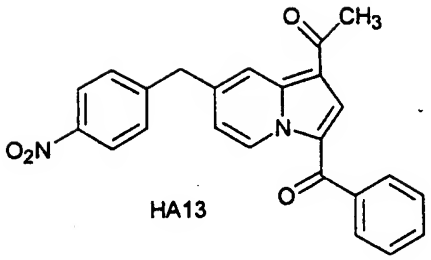
22. The method according to claim 15 wherein the compound causes the fragmentation of DNA in a Bcl-2 transfected HL-60 cell line when incubated with such cells at a concentration of not more than 100 μM for 24 hours.

23. The method according to claim 15 wherein the cells induced to undergo apoptosis comprise cancer cells.

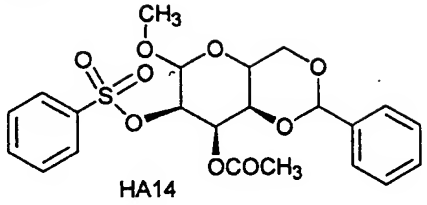
24. The method according to claim 15 wherein the cells induced to undergo apoptosis comprise virus-infected cells.

25. The method according to claim 15 wherein the cells induced to undergo apoptosis comprise self-reactive lymphocytes.

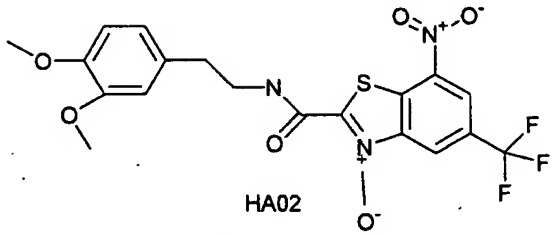
26. A method of inducing apoptosis of cells in a subject which are regulated by Bcl-2 comprising administering to the subject an effective amount of a compound selected from the group consisting of compounds HA13, HA14, HA02, HA03 and HA04:



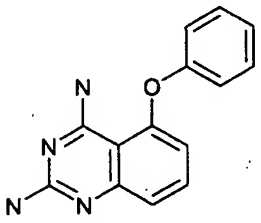
HA13



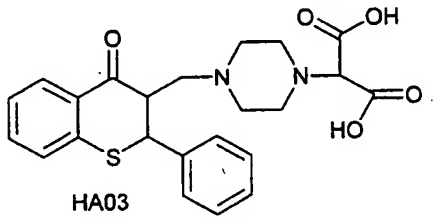
HA14



HA02



HA04



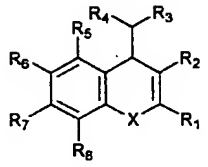
HA03

27. The method according to claim 26 wherein the cells induced to undergo apoptosis comprise cancer cells.

28. The method according to claim 26 wherein the cells induced to undergo apoptosis comprise virus-infected cells.

29. The method according to claim 26 wherein the cells induced to undergo apoptosis comprise self-reactive lymphocytes.

30. A method of inducing apoptosis of cells in a subject, which cells are regulated by Bcl-2, comprising administering to the subject an effective amount of an active compound of the formula III:



III

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; NCH_3; O; and S;

R_1 is selected from the group consisting of OH; NH_2; CHO; $COCH_3$; COOH; $N(C_{1-3}$ alkyl$)_2$; $NH(C_{1-3}$ alkyl); $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $N(C_{1-3}$alkyl$)_2$; $NH(C_{1-3}$ alkyl); and five- and six-member heterocyclic rings;

R_2 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; OH; NH_2; CHO; $COCH_3$; $OCOCH_3$; $OCOCH_2CH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$;

R_3 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; COOH; $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $CH{=}CH_2$; $CH_2CH{=}CH_2$; CH_2CHO; and five- and six-member heterocyclic rings;

R_4 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; $COCH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$; $OCOCH_3$; $OCOCH_2CH_3$;

R_5 is selected from the group consisting of hydrogen CH_3; OCH_3; OH: NH_2; Br; Cl; and F; and

R_6, R_7 and R_8 are selected from the group consisting of hydrogen, CH_3; CH_2CH_3; CF_3; NH_2; OH; OCH_3; CN; NO_2; Cl; Br; F; COOH; and $COOCH_3$; provided, at least one member of the group R_6, R_7 or R_8 must be Cl, Br or F when the remaining members of said group are hydrogen;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

31. The method according to claim 30 wherein the compound has a dissociation constant of not more than about 500 μM for binding the hydrophobic pocket on the Bcl-2 protein formed by the BH1, BH2, and BH3 domains of the Bcl-2 protein.

32. The method according to claim 30 wherein R_1 and R_3 are selected from the group consisting of piperidinyl, piperazinyl, morpholino, pyrimidyl, pyrrolyl, pyrrolidino and imidazyl.

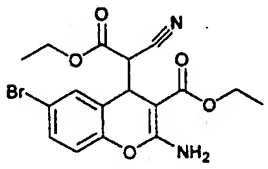
33. The method according to claim 30 wherein R_2 and R_4 are selected from the group consisting of $COCH_3$; $OCOCH_3$; $OCOCH_2CH_3$; COOH; $COOCH_3$; $COOCH_2CH_3$; and $COOCH_2CH_2CH_3$.

34. The method according to claim 30 wherein:

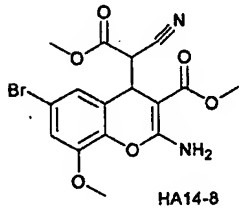
R_5 is selected from the group consisting of hydrogen, Br; Cl; and F;

R_6, R_7 R_8 are independently selected from the group consisting of NH_2; OH; OCH_3; CN; NO_2; Cl; Br; F.

35. The method according to claim 30 wherein the compound is selected from the group consisting of HA14-1 and HA14-8:



HA14-1



HA14-8

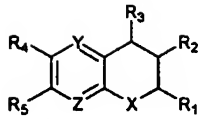
36. The method according to claim 30 wherein the compound causes the fragmentation of DNA in a Bcl-2 transfected HL-60 cell line when incubated with such cells at a concentration of not more than 100 μM for 24 hours.

37. The method according to claim 30 wherein the cells induced to undergo apoptosis comprise cancer cells.

38. The method according to claim 30 wherein the cells induced to undergo apoptosis comprise virus-infected cells.

39. The method according to claim 30 wherein the cells induced to undergo apoptosis comprise self-reactive lymphocytes.

40. A method of reversing Bcl-2-mediated blockage of apoptosis in cancer cells comprising contacting said cells with a compound of the formula I:



I

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; O; and S;

Y and Z are independently selected from the group consisting of CH and N; and when Z is N, then Y may further be $-CR_6$, where R_6 is selected from the group consisting of CH_3; OCH_3; CNH_2; and COH;

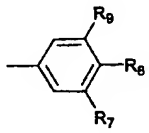
R_1 is selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; $NH(C_{1-3}$ alkyl); $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$;phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings;

R_2 is selected from the group consisting of hydrogen; C_{1-3} alkyl; C_{1-3} alkoxy; halogen; CF_3; NH_2; OH; COOH; $COOCH_3$; $CONH_2$; and $CONHCH_3$;

or, R_1 and R_2 together may form the group $-CH_2CH_2CH_2-$ or $-CH_2CH_2CH_2CH_2-$;

or, R_1 and R_2 together may form, starting from R_1, the group $-NHCH_2CH_2-$, $-NHCOCH_2-$, or $-OCOCH_2-$;

R_3 is selected from the group consisting of hydrogen; CH_3; CF_3; OCH_3; NH_2; OH; COOH; $COCH_3$; $CH{=}CH_2$; $CH_2{=}CHCH_2$; $CH(CH_3)_2$; CH_2OH; CH_2NH_2; CH_2COOH; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, OCH_3 or CF_3; five- and six-member heterocyclic rings; and a substituted phenyl group of the formula:



wherein

R_7, R_8 and R_9 are independently selected from the group consisting of hydrogen, CH_3, CF_3, OH, OCH_3, CH_2OH and CHO; provided that at least two of the members of the group R_7, R_8 and R_9 must be OH or OCH_3 when the remaining member of the group is hydrogen, CH_3 or CF_3;

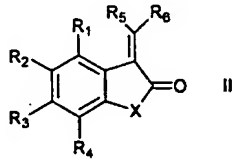
R_4 and R_5 are independently selected from the group consisting of hydrogen, CH_3, and OCH_3; and when Y and Z are both CH, R_4 and R_5 may be further selected from OH and NH_2;

or, R_4 and R_5 together may form the group $-CH_2CH_2CH_2-$ or $-CH_2CH_2CH_2CH_2-$;

or, R_4 and R_5 together may form, starting from R_4, the group $-NHCH_2CH_2-$, $-NHCOCH_2-$, $-OCOCH_2-$ or $-O(CH_2)_nO-$, wherein n is 1, 2 or 3;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

41. A method of reversing Bcl-2-mediated blockage of apoptosis in cancer cells comprising contacting said cells with a compound of the formula II:



II

wherein

R_1, R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; and $NH(C_{1-3}$ alkyl); and one of R_1, R_2, R_3 and R_4 may be phenyl or a heterocyclic ring; provided at least one of R_1, R_2, R_3 and R_4 must be hydrogen;

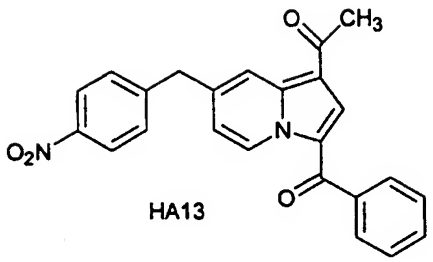
R_5 and R_6 are independently selected from the group consisting of hydrogen; CN; CH_2CN; $COOCH_3$; $CONH_2$; phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CH_3, OCH_3, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings; provided, only one of R_5 or R_6 may be phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic in the same compound, and further provided

that when one of R_5 or R_6 is phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic, then the other must be hydrogen;

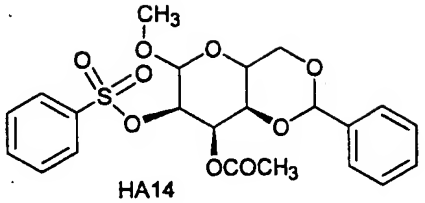
or at least one of R_5 and R_6 may be halogen, provided that the other must be C_{1-5} alkyl or C_{1-5} alkoxy.

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

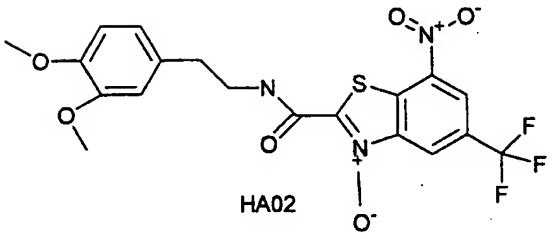
42. A method of reversing Bcl-2-mediated blockage of apoptosis in cancer cells comprising contacting said cells with a compound selected from the group consisting of compounds HA13, HA14, HA02, HA03 and HA04:



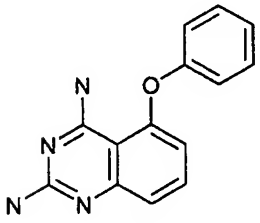
HA13



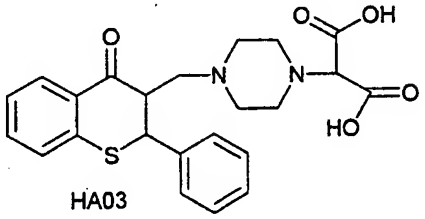
HA14



HA02

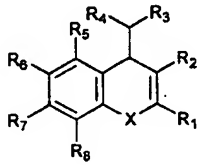


HA04



HA03

43. A method of reversing Bcl-2-mediated blockage of apoptosis in cancer cells comprising contacting said cells with a compound of the formula III:



III

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; NCH_3; O; and S;

R_1 is selected from the group consisting of OH; NH_2; CHO; $COCH_3$; COOH; $N(C_{1-3}\ alkyl)_2$; $NH(C_{1-3}\ alkyl)$; $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $N(C_{1-3}alkyl)_2$; $NH(C_{1-3}\ alkyl)$; and five- and six-member heterocyclic rings;

R_2 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; OH; NH_2; CHO; $COCH_3$; $OCOCH_3$; $OCOCH_2CH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$;

R_3 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; COOH; $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $CH{=}CH_2$; $CH_2CH{=}CH_2$; CH_2CHO; and five- and six-member heterocyclic rings;

R_4 is selected from the group consisting of C_{1-3} alkyl; C_{1-3}alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; $COCH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$; $OCOCH_3$; $OCOCH_2CH_3$;

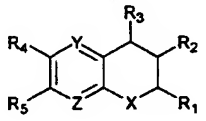
R_5 is selected from the group consisting of hydrogen CH_3; OCH_3; OH: NH_2; Br; Cl; and F; and

R_6, R_7 and R_8 are selected from the group consisting of hydrogen, CH_3; CH_2CH_3; CF_3; NH_2; OH; OCH_3; CN; NO_2; Cl; Br; F; COOH; and $COOCH_3$; provided, at least one member

of the group R_6, R_7 or R_8 must be Cl, Br or F when the remaining members of said group are hydrogen;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

44. A method for treating a subject afflicted with a cancer characterized by cancer cells which express Bcl-2 comprising administering to the subject an effective amount of a compound of the formula I:



I

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; O; and S;

Y and Z are independently selected from the group consisting of CH and N; and when Z is N, then Y may further be $-CR_6$, where R_6 is selected from the group consisting of CH_3; OCH_3; CNH_2; and COH;

R_1 is selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; $NH(C_{1-3}$ alkyl); $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$;phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-

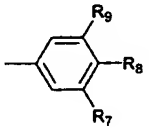
substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings;

R_2 is selected from the group consisting of hydrogen; C_{1-3} alkyl; C_{1-3} alkoxy; halogen; CF_3; NH_2; OH; COOH; $COOCH_3$; $CONH_2$; and $CONHCH_3$;

or, R_1 and R_2 together may form the group -$CH_2CH_2CH_2$- or -$CH_2CH_2CH_2CH_2$-;

or, R_1 and R_2 together may form, starting from R_1, the group -$NHCH_2CH_2$-, -$NHCOCH_2$-, or -$OCOCH_2$-;

R_3 is selected from the group consisting of hydrogen; CH_3; CF_3; OCH_3; NH_2; OH; COOH; $COCH_3$; $CH=CH_2$; $CH_2=CHCH_2$; $CH(CH_3)_2$; CH_2OH; CH_2NH_2; CH_2COOH; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, OCH_3 or CF_3; five- and six-member heterocyclic rings; and a substituted phenyl group of the formula:



wherein

R_7, R_8 and R_9 are independently selected from the group consisting of hydrogen, CH_3, CF_3, OH, OCH_3, CH_2OH and CHO; provided that at least two of the members of the group R_7, R_8 and R_9 must be OH or OCH_3 when the remaining member of the group is hydrogen, CH_3 or CF_3;

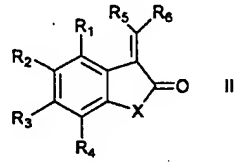
R_4 and R_5 are independently selected from the group consisting of hydrogen, CH_3, and OCH_3; and when Y and Z are both CH, R_4 and R_5 may be further selected from OH and NH_2;

or, R_4 and R_5 together may form the group -$CH_2CH_2CH_2$- or -$CH_2CH_2CH_2CH_2$-;

or, R_4 and R_5 together may form, starting from R_4, the group -$NHCH_2CH_2$-, -$NHCOCH_2$-, -$OCOCH_2$- or -$O(CH_2)_nO$-, wherein n is 1, 2 or 3;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

45. A method for treating a subject afflicted with a cancer characterized by cancer cells which express Bcl-2 comprising administering to the subject an effective amount of a compound of the formula II:



II

wherein

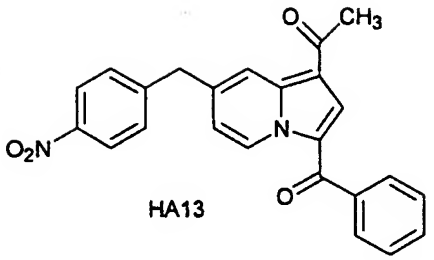
R_1, R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; and $NH(C_{1-3}$ alkyl); and one of R_1, R_2, R_3 and R_4 may be phenyl or a heterocyclic ring; provided at least one of R_1, R_2, R_3 and R_4 must be hydrogen;

R_5 and R_6 are independently selected from the group consisting of hydrogen; CN; CH_2CN; $COOCH_3$; $CONH_2$; phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CH_3, OCH_3, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings; provided, only one of R_5 or R_6 may be phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic in the same compound, and further provided that when one of R_5 or R_6 is phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic, then the other must be hydrogen;

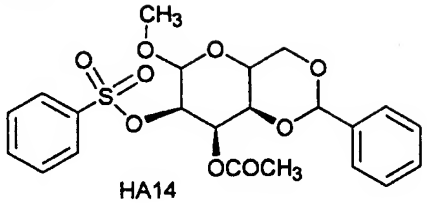
or at least one of R_5 and R_6 may be halogen, provided that the other must be C_{1-5} alkyl or C_{1-5} alkoxy.

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

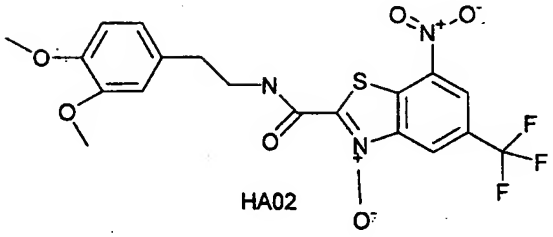
46. A method for treating a subject afflicted with a cancer characterized by cancer cells which express Bcl-2 comprising administering to the subject an effective amount of a compound selected from the group consisting of compounds HA13, HA14, HA02, HA03 and HA04:



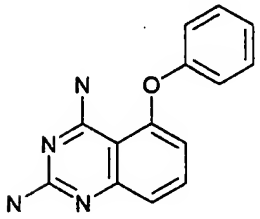
HA13



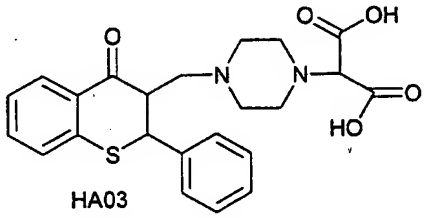
HA14



HA02

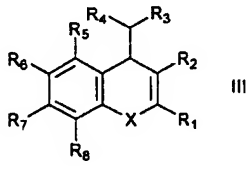


HA04



HA03

47. A method for treating a subject afflicted with a cancer characterized by cancer cells which express Bcl-2 comprising administering to the subject an effective amount of a compound of the formula III:



III

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; NCH_3; O; and S;

R_1 is selected from the group consisting of OH; NH_2; CHO; $COCH_3$; COOH; $N(C_{1-3}\ alkyl)_2$; $NH(C_{1-3}\ alkyl)$; $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $N(C_{1-3}alkyl)_2$; $NH(C_{1-3}\ alkyl)$; and five- and six-member heterocyclic rings;

R_2 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; OH; NH_2; CHO; $COCH_3$; $OCOCH_3$; $OCOCH_2CH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$;

R_3 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; COOH; $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $CH{=}CH_2$; $CH_2CH{=}CH_2$; CH_2CHO; and five- and six-member heterocyclic rings;

R_4 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; $COCH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$; $OCOCH_3$; $OCOCH_2CH_3$;

R_5 is selected from the group consisting of hydrogen CH_3; OCH_3; OH: NH_2; Br; Cl; and F; and

R_6, R_7 and R_8 are selected from the group consisting of hydrogen, CH_3; CH_2CH_3; CF_3; NH_2; OH; OCH_3; CN; NO_2; Cl; Br; F; COOH; and $COOCH_3$; provided, at least one member of the group R_6, R_7 or R_8 must be Cl, Br or F when the remaining members of said group are hydrogen;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

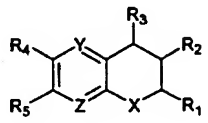
48. A method according to claim 44 wherein the cancer is selected from the group of cancers consisting of prostate, colorectal, gastric, non-small lung, renal and thyroid cancers, neuroblastoma, melanoma, and acute and chronic lymphocytic and non-lymphocytic leukemia.

49. A method according to claim 45 wherein the cancer is selected from the group of cancers consisting of prostate, colorectal, gastric, non-small lung, renal and thyroid cancers, neuroblastoma, melanoma, and acute and chronic lymphocytic and non-lymphocytic leukemia.

50. A method according to claim 46 wherein the cancer is selected from the group of cancers consisting of prostate, colorectal, gastric, non-small lung, renal and thyroid cancers, neuroblastoma, melanoma, and acute and chronic lymphocytic and non-lymphocytic leukemia.

51. A method according to claim 47 wherein the cancer is selected from the group of cancers consisting of prostate, colorectal, gastric, non-small lung, renal and thyroid cancers, neuroblastoma, melanoma, and acute and chronic lymphocytic and non-lymphocytic leukemia.

52. A method for treating a subject for an autoimmune disorder comprising administering to the subject an effective amount of a compound of the formula I:



I

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; O; and S;

Y and Z are independently selected from the group consisting of CH and N; and when Z is N, then Y may further be $-CR_6$, where R_6 is selected from the group consisting of CH_3; OCH_3; CNH_2; and COH;

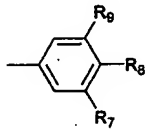
R_1 is selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; $NH(C_{1-3}$ alkyl); $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$;phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings;

R_2 is selected from the group consisting of hydrogen; C_{1-3} alkyl; C_{1-3} alkoxy; halogen; CF_3; NH_2; OH; COOH; $COOCH_3$; $CONH_2$; and $CONHCH_3$;

or, R_1 and R_2 together may form the group -$CH_2CH_2CH_2$- or -$CH_2CH_2CH_2CH_2$-;

or, R_1 and R_2 together may form, starting from R_1, the group -$NHCH_2CH_2$-, -$NHCOCH_2$-, or -$OCOCH_2$-;

R_3 is selected from the group consisting of hydrogen; CH_3; CF_3; OCH_3; NH_2; OH; COOH; $COCH_3$; $CH=CH_2$; $CH_2=CHCH_2$; $CH(CH_3)_2$; CH_2OH; CH_2NH_2; CH_2COOH; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, OCH_3 or CF_3; five- and six-member heterocyclic rings; and a substituted phenyl group of the formula:



wherein

R_7, R_8 and R_9 are independently selected from the group consisting of hydrogen, CH_3, CF_3, OH, OCH_3, CH_2OH and CHO; provided that at least two of the members of the group R_7, R_8 and R_9 must be OH or OCH_3 when the remaining member of the group is hydrogen, CH_3 or CF_3;

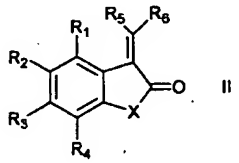
R_4 and R_5 are independently selected from the group consisting of hydrogen, CH_3, and OCH_3; and when Y and Z are both CH, R_4 and R_5 may be further selected from OH and NH_2;

or, R_4 and R_5 together may form the group -$CH_2CH_2CH_2$- or -$CH_2CH_2CH_2CH_2$-;

or, R_4 and R_5 together may form, starting from R_4, the group -$NHCH_2CH_2$-, -$NHCOCH_2$-, -$OCOCH_2$- or -$O(CH_2)_nO$-, wherein n is 1, 2 or 3;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

53. A method for treating a subject for an autoimmune disorder comprising administering to the subject an effective amount of a compound of the formula II:



II

wherein

R_1, R_2, R_3 and R_4 are independently selected from the group consisting of hydrogen; C_{1-5} alkyl; C_{1-5} alkoxy; OH; NH_2; NO_2; CHO; $COCH_3$; COOH; $COOCH_3$; $N(C_{1-3}$ alkyl$)_2$; and $NH(C_{1-3}$ alkyl); and one of R_1, R_2, R_3 and R_4 may be phenyl or a heterocyclic ring; provided at least one of R_1, R_2, R_3 and R_4 must be hydrogen;

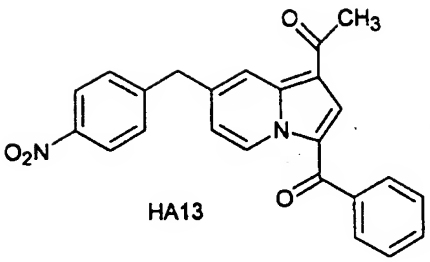
R_5 and R_6 are independently selected from the group consisting of hydrogen; CN; CH_2CN; $COOCH_3$; $CONH_2$; phenyl; phenyl which is mono-, di-, or tri-substituted with NH_2, OH, halogen, NO_2, CH_3, OCH_3, CF_3, COOH or $COOCH_3$; cyclohexyl; cyclohexyl which is mono-, di-, or tri-substituted

with NH_2, OH, halogen or CF_3; and five- and six-member heterocyclic rings; provided, only one of R_5 or R_6 may be phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic in the same compound, and further provided that when one of R_5 or R_6 is phenyl, substituted phenyl, cyclohexyl, substituted cyclohexyl or heterocyclic, then the other must be hydrogen;

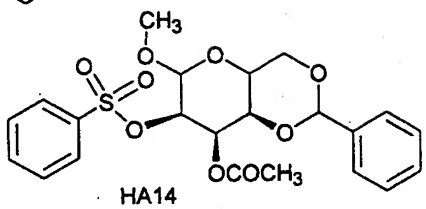
or at least one of R_5 and R_6 may be halogen, provided that the other must be C_{1-5} alkyl or C_{1-5} alkoxy.

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

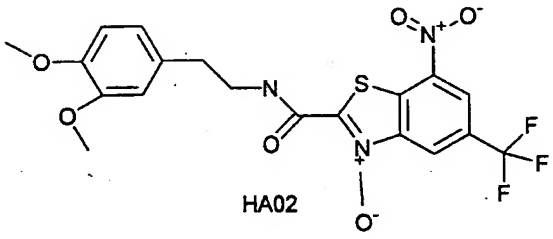
54. A method for treating a subject for an autoimmune disorder comprising administering to the subject an effective amount of a compound selected from the group consisting of compounds HA13, HA14, HA02, HA03 and HA04:



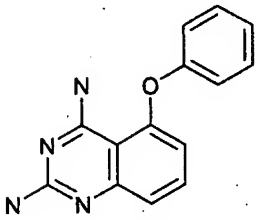
HA13



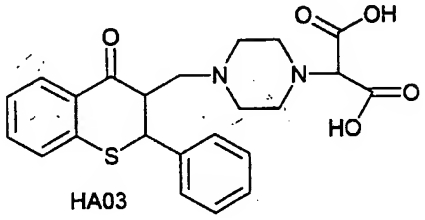
HA14



HA02

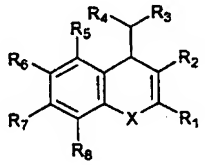


HA04



HA03

55. A method for treating a subject for an autoimmune disorder comprising administering to the subject an effective amount of a compound of the formula III:



III

wherein:

X is selected from the group consisting of CH_2; $CHOCH_3$; NH; NCH_3; O; and S;

R_1 is selected from the group consisting of OH; NH_2; CHO; $COCH_3$; COOH; $N(C_{1-3}$ alkyl$)_2$; $NH(C_{1-3}$ alkyl); $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $N(C_{1-3}$alkyl$)_2$; $NH(C_{1-3}$ alkyl); and five- and six-member heterocyclic rings;

R_2 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; OH; NH_2; CHO; $COCH_3$; $OCOCH_3$; $OCOCH_2CH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$;

R_3 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; COOH; $OCOCH_3$; $OCOCH_2CH_3$; $NHCOCH_3$; $NHNHCOCH_3$; $NHNHCONH_2$; $CH=CH_2$; $CH_2CH=CH_2$; CH_2CHO; and five- and six-member heterocyclic rings;

R_4 is selected from the group consisting of C_{1-3} alkyl; C_{1-3} alkoxy; CN; CH_2CN; CH_2NO_2; CHO; $COCH_3$; $COCH_3$; COOH; $COOCH_3$;$COOCH_2CH_3$; $COOCH_2CH_2CH_3$; $OCOCH_3$; $OCOCH_2CH_3$;

R_5 is selected from the group consisting of hydrogen CH_3; OCH_3; OH: NH_2; Br; Cl; and F; and

R_6, R_7 and R_8 are selected from the group consisting of hydrogen, CH_3; CH_2CH_3; CF_3; NH_2; OH; OCH_3; CN; NO_2; Cl;

Br; F; COOH; and $COOCH_3$; provided, at least one member of the group R_6, R_7 or R_8 must be Cl, Br or F when the remaining members of said group are hydrogen;

or a pharmaceutically acceptable salt thereof when the compound includes at least one NH_2 or COOH substituent.

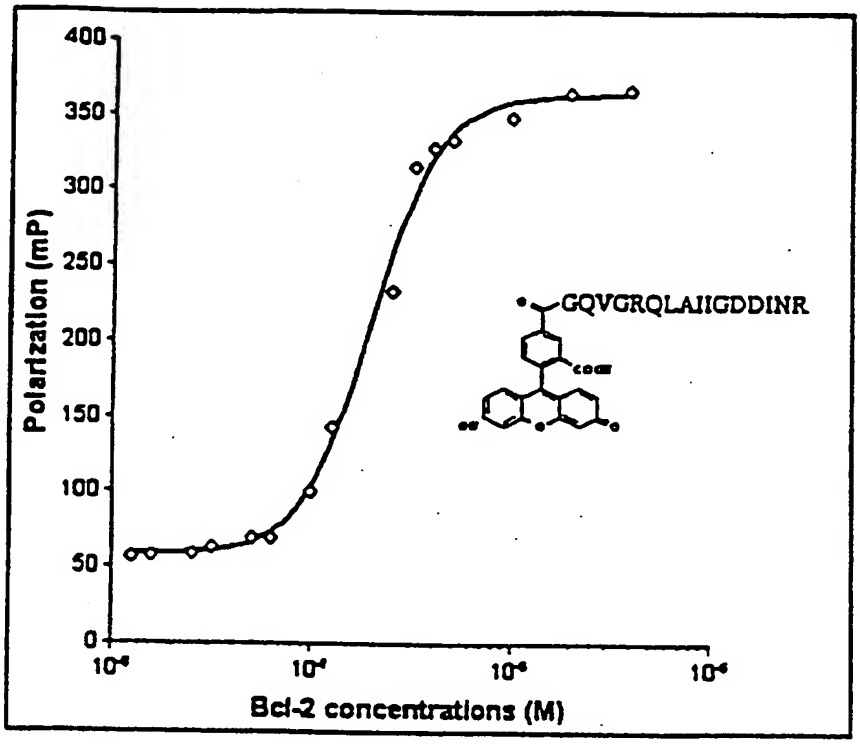

400
350
300
250
200
150
100
50
0
Polarization (mP)
GQVGRQLAIIGDDINR
Bcl-2 concentrations (M)

FIG. 1

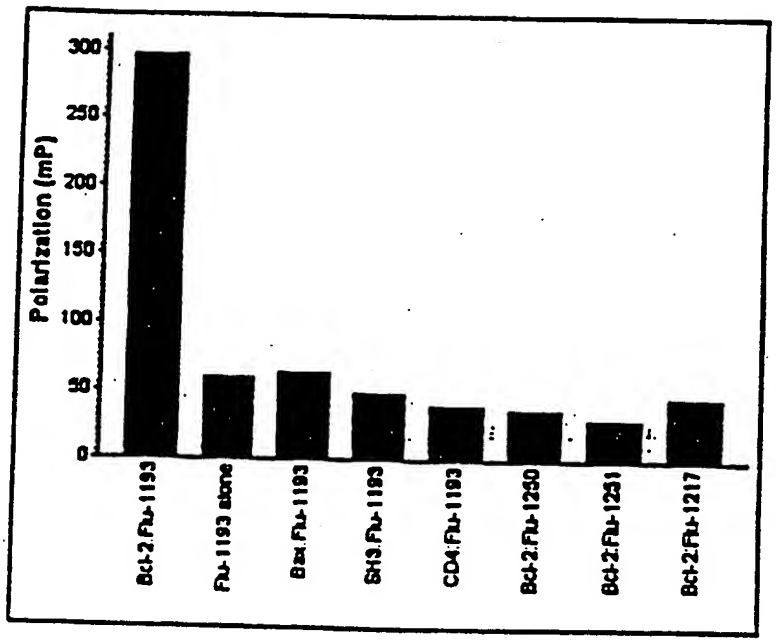

Polarization (mP)
300
250
200
150
100
50
0
Bcl-2:Flu-1193
Flu-1193 alone
Bax:Flu-1193
BH3:Flu-1193
CD4:Flu-1193
Bcl-2:Flu-1250
Bcl-2:Flu-1251
Bcl-2:Flu-1217

FIG. 2


0 1 2 3

FIG. 3

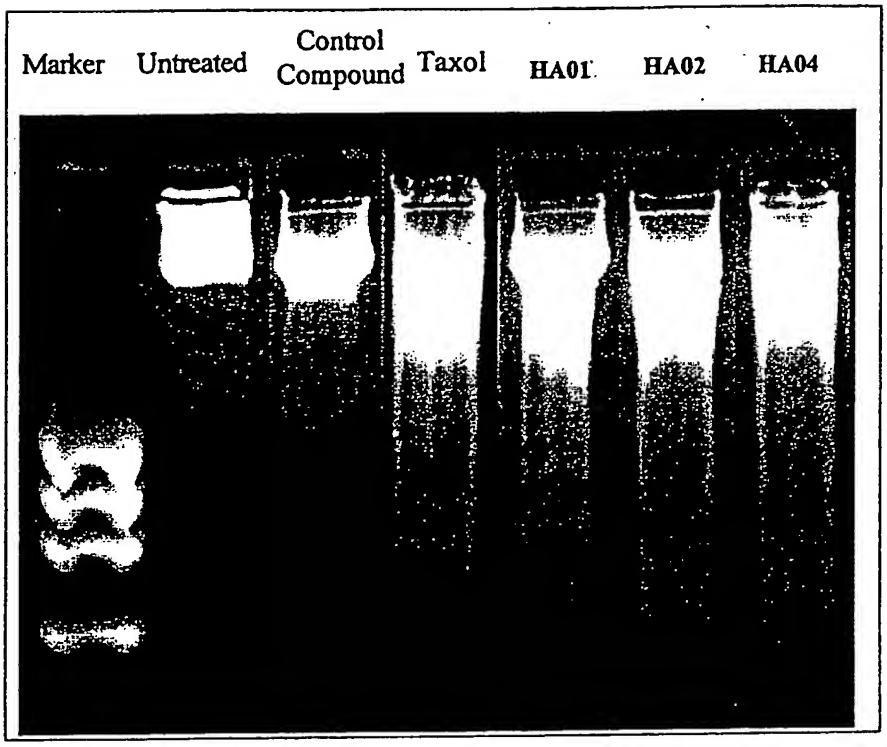


FIG. 4

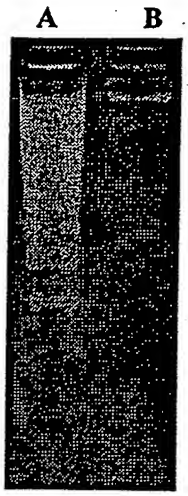


FIG. 5

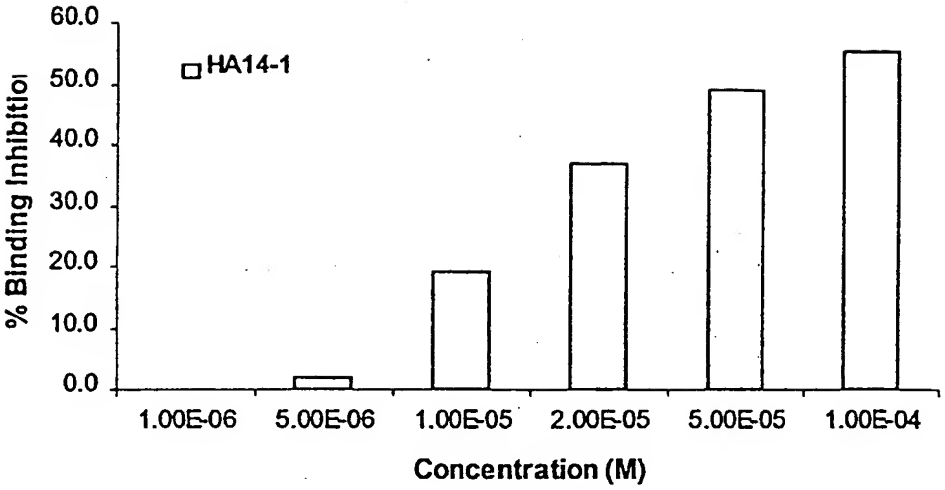

60.0
50.0
40.0
30.0
20.0
10.0
0.0
% Binding Inhibitio
HA14-1
1.00E-06
5.00E-06
1.00E-05
2.00E-05
5.00E-05
1.00E-04
Concentration (M)

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12384

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/35
US CL :514/453, 454, 455, 456
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/454, 456

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST: Bcl-2, cancer, inhibition

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,028,606 A (VENET et al.) 02 July 1991, see entire document	1-55

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 NOVEMBER 1999	Date of mailing of the international search report 06 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer THEODORE J. CRIARES CV JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX Telephone No. (703) 308-1235

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